UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

FACULDADE DE MEDICINA – Campus de Botucatu

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Migração e invasão celular *in vitro* de células humanas expressando variantes da proteína LMP1 do vírus de Epstein-Barr (EBV)

Dissertação apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Mestre em Patologia.

Orientador: Prof. Dr. Deilson Elgui de Oliveira

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Palavras-chave: Carcinoma de nasofaringe (NPC); Invasão celular; Migração celular; Proteína latente de membrana - LMP1; vírus de Epstein-Barr (EBV).

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Dedico esta dissertação com todo meu amor:

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"De tudo, ficaram três coisas:

A certeza de que estamos sempre começando...

A certeza de que precisamos continuar...

A certeza de que seremos interrompidos antes de terminar...

Portanto devemos:

Fazer da interrupção um caminho novo ...

Da queda, um passo de dança...

Do medo, uma escada...

Do sonho, uma ponte...

Da procura, um encontro..."

Fernando Sabino

Resumo

O vírus de Epstein-Barr (*Epstein-Barr virus - EBV*) infecta latentemente mais de 90% da população humana. A infecção viral está associada ao desenvolvimento de alguns cânceres, incluindo o carcinoma de nasofaringe. Alguns estudos sugerem a contribuição na evasão imune e progressão dos cânceres causados pelo EBV. Diferentes variantes da proteína latente de membrana 1 (LMP1), principal produto do EBV, são discriminadas, principalmente, por variações nos domínios transmembrana e carboxi-terminais da proteína. O domínio C-terminal de LMP1 é capaz de ativar vias de sinalização intracelular que regulam os fenômenos de migração e invasão celular. Adicionalmente, induz síntese de produtos que degradam a matriz extracelular e favorecem a angiogênese. Até o momento, não se sabe se diferentes variantes de LMP1 apresentam propriedades distintas no que se refere a esses fenômenos do escape imune e progressão tumoral. Assim sendo, no presente estudo avaliamos a influência das variantes de LMP1 sobre a capacidade de migração e invasão celular *in vitro* e a imunomodulação de HLA-ABC e HLA-DR, CD80, CD83, CD54, CD40 e PD-L1 nas linhagens celulares HEK293T e NP69. Observamos que as variantes Alaskan, Med+, China 1 e China 2 induziram a migração celular da linhagem NP69 de maneira distinta quando comparadas entre si e com a célula sem LMP1. Observamos, também, maior capacidade de invasão celular das células HEK293T-China 2 em comparação à HEK293T. Com relação à expressão de moléculas envolvidas na modulação da resposta imune, observamos que a transfecção de LMP1 aumentou a expressão de CD80 e CD83 apenas nas células NP69-Alaskan, e de CD54 e PD-L1 de maneira similar nas células NP69-LMP1. Conclui-se, portanto, que LMP1 promove migração distinta das células NP69-LMP1 e invasão de células HEK293T-China 2, e modula a expressão de moléculas envolvidas no escape imunológico.

Palavras-chave: EBV, LMP1, NPC, Migração e Invasão celular.

Abstract

The Epstein-Barr virus (EBV) latently infects more than 90% of human adults. Viral infection is associated to the development of some cancers, including nasopharyngeal carcinoma. Oncogenic potential of the virus is usually studied in terms of its capacity to transform the infected cells nevertheless some studies suggest that the EBV infection may also contribute to immune evasion, and cancer progression. Several latent membrane protein 1 (LMP1) variants are described, discriminated mainly by variations in its C-terminal and the transmembrane domains of the protein. LMP1 C-terminal domain activate intracellular signaling pathways that regulated cell migration; moreover, LMP1 upregulate the expression of cell proteins with roles in extracellular matrix remodeling and angiogenesis. Currently it is unknown whether different LMP1 variants possess distinct properties regarding biological phenomena relevant to immune evasion, and cancer progression. Thus, in the present study we evaluated the *in vitro* migration and invasiveness, and immunomodulation of HLA-ABC, HLA-DR, CD80, CD83, CD54, CD40, and PD-L1 of HEK293T cells, and NP69 cells. We observed that Alaskan, Med+, China 1, and China 2 stimulates distinctly migration of NP69 when compared to cell without LMP1. We also saw that HEK293T-China 2 had a pronounced cellular invasion when compared to HEK293T. In regard of expression of molecules involved in immune response modulation, we observed that LMP1 enhanced expression of CD80, and CD83 in NP69-Alaskan cells; and CD54, and PD-L1 in a similar level in NP69-LMP1 cells. In summary, LMP1 promote distinct cellular migration in NP69LMP1 cells, and HEK293T-China 2 invasion, and is capable of modulate molecules involved in immune evasion.

Keywords: EBV, LMP1, NPC, Cellular migration, and cellular invasion.

Lista de siglas

BARF1	Transcritos da região BamHIA do EBV
BL	*Burkitt's lymphoma*
CDK1 e 2	Inibidor de quinase dependente de ciclina 1 e 2
cHL	*Classical Hodgkin lymphoma*
COX-2	Ciclooxigenase 2
CTAR1, 2 e 3	Região ativadora carboxi-terminal 1, 2 e 3
DMEM	Meio modificado de Dulbecco Eagle
EBER	*Small non-polyadenilated RNAs*
EBNA 2, 3A-C	Antígenos nucleares do EBV 2, 3A-C
EBV	*Epstein-Barr virus*
EGFR	Receptor do fator de crescimento epidermal
FGF-2	Fator de crescimento de fibroblastos
GC	*Gastric carcinoma*
HEK293T	Linhagem celular embrionária de rim
HIF1-α	Fator transcricional pró-metastático 1 alpha
HLA	*Human Leukocyte Antigen*
IL-8	Interleucina 8
IM	*Infectious mononucleosis*
Keratinocyte-SFM	Meio de queratinócito livre de soro
LMP1	Proteína Latente de membrane 1
MMP 1, 3 e 9	Metaloproteinases de matriz 1, 3 e 9
NF-κB	Fator nuclear kappa B
NPC	*Nasopharyngeal carcinoma*
NP69	Linhagem celular imortalizada de nasofaringe
PI3K-Akt	Fosfatidilinositol 3 quinase-Akt
PTLD	*Post-transplant Lymphoproliferative Disorders*
TNFAIP2	Proteína 2 induzida pelo fator de necrose tumoral alpha
VEGF	Fator de crescimento do endotélio vascular

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Sumário

Capítulo 1: Revisão da Literatura

1. Introdução

A primeira descrição do vírus Epstein-Barr (EBV*)*, também denominado Herpesvírus Humano tipo 4 (HHV4), ocorreu em 1964 por Epstein e colaboradores, a partir da análise por microscopia eletrônica de linhagens celulares isoladas de um linfoma de primeira infância muito comum na África – o Linfoma de Burkitt (BL)(1). O EBV infecta latentemente mais de 90% da população humana adulta, sendo que, na maioria dos casos, a infecção primária ocorre entre a infância e a adolescência(2). A infecção pelo EBV se associa ao desenvolvimento da mononucleose infecciosa (IM*)*(3)*,* além de uma variedade de doenças neoplásicas humanas, tais como carcinoma de nasofaringe (NPC*)*(4), o linfoma de Hodgkin clássico (cHL) e desordens linfoproliferativas pós-transplante (PTLD*)*(5).

1.1. Biologia do vírus de Epstein-Barr

O EBV pertence à família *Herpesviridae*, subfamília *Gammaherpesvirinae*, gênero *Lymphocryptovirus*(6). A partícula viral é icosaédrica e possui um nucleocapsídeo constituído por 162 capsômeros e um envelope externo herpesviral típico, contendo as glicoproteínas gp350/220, gp110 e gp85(7). O genoma viral é linear e constituído por molécula de DNA dupla fita de 172kb. Possui 0,5kb de repetições diretas terminais e 3kb de repetições diretas internas(8), codificando mais de 85 genes(9). De acordo com polimorfismos presentes nos genes virais EBNA2(10), EBNA3A, EBNA3B e EBNA3C(11), são descritos dois genótipos principais do EBV, denominados EBV-1 e EBV-2.

A primeira infecção pelo EBV ocorre por via oral e o primeiro sítio de replicação viral é o epitélio da orofaringe, no qual o vírus pode estabelecer infecção persistente. Sua replicação nas células epiteliais promove produção e liberação de novas partículas virais, que são capazes de infectar novas células susceptíveis no organismo, como os linfócitos B, ou serem transmitidas a novos hospedeiros(12).

O ciclo biológico do EBV é tradicionalmente dividido em dois estágios, os ciclos latente e lítico. No ciclo latente, o genoma viral é mantido em número constante de cópias e há expressão de um número restrito de genes virais, de acordo com

diferentes programas de latência, denominados latência tipo I, tipo II e tipo III (Quadro 1)(13). Admite-se que essas variações na expressão gênica viral possibilitem a adaptação do vírus a diferentes condições do hospedeiro, ocasionalmente desencadeando doenças(14). No ciclo lítico, o genoma do EBV é replicado e novas partículas virais são produzidas para sua disseminação no organismo e contágio de novos hospedeiros(15).

Quadro 1: Programas de latência na infecção pelo EBV.

Programas	Genes virais expressos	Principais doenças
Latência I (programa restrito)	EBNA1, EBERs, BARF1	BL, IM, GC
Latência II (programa padrão)	EBNA1, LMP1, LMP2A, LMP2B, EBERs, BARF1	NPC, cHL
Latência III (programa de crescimento)	EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, LMP1, LMP2, EBERs	PTLD

A infecção de linfócitos B pelo EBV se dá pela ligação do vírus à molécula CD21 na superfície celular por meio de sua principal glicoproteína de envelope, a gp350(16). A entrada do vírus requer interação de uma segunda glicoproteína, a gp42, com moléculas de classe II do complexo principal de histocompatibilidade humano (HLA), que atua como um co-receptore. A infecção de células epiteliais, por sua vez, ocorre por 2 mecanismos distintos (I) contato direto célula-célula na membrana apical da célula epitelial e (II) entrada basolateral de vírus livres. No último, a glicoproteína BMRF-2 do EBV interage com a β_1-integrina e $\alpha_5\beta_1$-integrina da superfície das células epiteliais da orofaringe, permitindo a entrada do vírus(17).

Durante a fase lítica do ciclo biológico do EBV, as células infectadas permissivas sofrem alterações como marginação do DNA e inibição da síntese de macromoléculas do hospedeiro, de modo a favorecer a síntese do DNA viral, a montagem dos nucleocapsídeos e o envelopamento do vírus(18,19). Nessa fase, são expressos RNA de mais de 80 genes, cujos transcritos podem ser discriminados de acordo com o momento em que surgem, sendo denominados produtos precoces (imediatamente precoces ou precoces) ou tardios(20). Após a montagem, as novas partículas virais são liberadas das células infectadas e se tornam capazes de infectar novas células susceptíveis(2).

Entre as neoplasias induzidas pelo EBV, o carcinoma de nasofaringe (NPC) é particularmente comum em algumas áreas da China e Sudeste da Ásia(21), em populações africanas do Mediterrâneo e Inuit do Alaska(22). Além da infecção pelo EBV, admite-se que fatores étnicos(23) e ambientais (incluindo componentes da dieta) devem contribuir em sua etiopatogenia(24,25).

Metade dos casos de linfoma de Hodgkin clássico (cHL) também está relacionada à infecção pelo EBV. Embora a patogenia da doença não esteja completamente elucidada, o cHL tem um componente importante de anormalidades relacionadas à resposta imunitária(26). Por fim, as desordens linfoproliferativas pós-transplante (PTLD) caracteristicamente se desenvolve em contextos de imunossupressão terapeuticamente induzida após transplante de órgãos sólidos ou transplante alogênico de medula óssea. A incidência é maior em crianças que em adultos, posto que crianças têm menor chance de exposição prévia ao EBV e são mais susceptíveis à infecção primária próximo ao período do transplante. Assim, a PTLD acomete mais frequentemente pacientes EBV-negativos no momento do transplante, que não apresentavam imunidade prévia ao vírus(27). Em comum, essas doenças tão distintas – o NPC, o cHL e a PTLD – apresentam pelo menos um aspecto biológico relacionado à infecção pelo EBV: a expressão da oncoproteína viral LMP-1, discutida a seguir.

1.2. Proteína latente de membrana 1 (LMP1) do EBV

A LMP1 é o principal produto do EBV com propriedades transformantes, atuando como um oncogene clássico tanto *in vivo* (e.g., pela transformação de linfócitos B)(28), quanto *in vitro* (e.g., transformação de fibroblastos de roedores)(29). É expressa em diferentes cânceres associados ao EBV e apresenta elevada capacidade de transformação dos linfócitos B(21,30), além de alterar a capacidade de diferenciação de linhagens epiteliais *in vitro*(31,32). Sua estrutura geral está representada na Figura 1A.

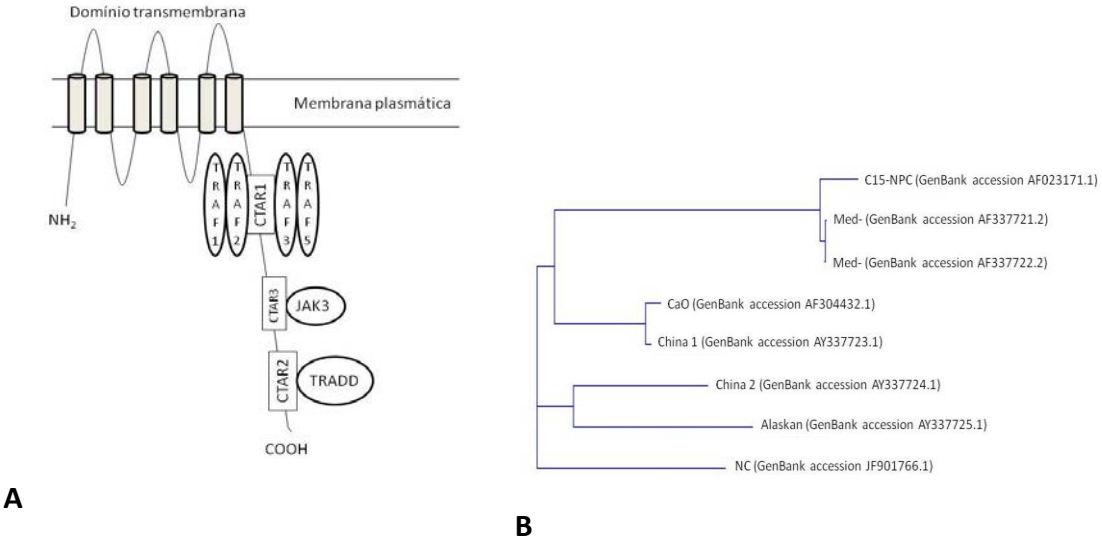

Figura 1: (A): Estrutura geral da oncoproteína LMP1 do vírus Epstein-Barr. (B) Relação filogenética das variantes de LMP1, desenhada utilizando-se o software Mega6 (33). As sequências de DNA foram obtidas na base de dados GenBank do Centro Nacional para Informação Biotecnológica (NCBI).

O domínio transmembrana de LMP1 é responsável pela oligomerização das moléculas à membrana plasmática, possibilitando que a proteína desempenhe papel de um receptor para fator de crescimento constitutivamente ativo(34). As regiões ativadoras do domínio carboxi-terminal, CTAR1 e CTAR2, são sítios de ligação a moléculas associadas aos receptores do fator de necrose tumoral (TNFr). Após ligação a esses fatores, LMP1 passa a efetuar sinalização intracelular semelhante à do receptor CD40 de células B e TNFr tipo II(35).

A região CTAR1 da LMP-1 localiza-se próxima à face citoplasmática da membrana plasmática e possui uma porção PXQXT (aminoácidos 204 e 208) que interage com ligantes do receptor de TNF (T*RAF*) TRAF1, TRAF2, TRAF3 e TRAF5(36,37). CTAR2, no extremo da região carboxi-terminal, liga a proteína de domínio de morte associada ao TNFr (TRADD) e a proteína interativa do receptor (RIP)(38). Mais recentemente, foi descrito também um domínio CTAR3 (aminoácidos 275 a 330), ligante da proteína transdutora de sinal *Janus Kinase 3 (JAK3)*(39).

LMP1 é capaz de ativar diversas vias de sinalização intracelular, com destaque para o fator nuclear-κB (NF-κB)(40,41), necessária para a transformação e sobrevivência de células B. Adicionalmente, ativa importantes moléculas transdutoras

de sinal como a proteína ativadora 1 (AP-1)(42), a quinase N-terminal c-Jun (JNK)(43), a fosfatiditilnositol 3 quinase-Akt (*PI3K-Akt)*(44) e STAT(45). Como efeitos da expressão de LMP1, observa-se também supraregulação de metaloproteinases de matriz 1 (MMP-1) e 9 (MMP-9), notadamente em células malignas(46,47); expressão de receptores do fator de crescimento epidermal (EGFR)(48), do fator de crescimento de endotélio vascular (VEGF)(49), além de induzir proteínas anti-apoptóticas como A20, bcl-2 e Mcl1 nas células B(50,51).

Além da ativação dessas vias de sinalização, LMP1 participa da imunomodulação de moléculas envolvidas na ativação de linfócitos T e apresentação de antígenos, como um dos mecanismos de escape imunológico. LMP1 promove a supraregulação das moléculas HLA-ABC e HLA-DR em células de NPC. Sabe-se que as diferentes variantes de LMP1 possuem alterações específicas em epítopos HLA de LMP1, permitindo, assim, a expressão de LMP1 nas células infectadas pelo EBV sem reconhecimento específico pelos linfócitos T citotóxicos (CTL)(52).

LMP1, por meio da ativação de NF-κB via CTAR1, supraregula a molécula co-estimulatória CD83(53). Embora essa molécula seja um importante marcador de células dendriticas maduras, sua expressão por células tumorais pode estar associada ao escape imunológico. A translocação da subunidade RelB de NF-κB mediada por CTAR1 de LMP1 promove a indução da expressão de outra molécula co-estimulatória, CD80(54). A expressão de CD54 (ICAM-1), importante molécula de adesão intercelular, pode ser supraregulada em NPC por LMP1(55).

A molécula CD40 é expressa em células epiteliais em estágios primários da diferenciação, além de participar da ativação de linfócitos T durante a resposta imunitária(56,57). Sabe-se que LMP1 mimetiza a ação da molécula CD40, principalmente, na ativação de vias intracelulares como NF-κB, que levam à sobrevivência e proliferação das células infectadas pelo EBV. Além de mimetizar essa molécula, LMP1 é, também, capaz de supraregular sua expressão em células de NPC(56).

Por fim, LMP1, também, participa da supraregulação da expressão de uma molécula imunossupressora - PD-L1 por meio da ativação de algumas vias intracelulares como JAK/STAT, AP-1 e NF-κB(58).

A análise de isolados de tumores EBV-positivos demonstrou que os genes de

LMP1 não diferem entre os genótipos 1 e 2 do vírus(59). Entretanto, sequências distintas de aminoácidos em LMP1 têm sido identificadas, permitindo a discriminação de tais variantes (32). Os estudos realizados para discriminação de variantes de LMP1, tomando como referência a proteína codificada no protótipo B95.8 do EBV-1(8) e pelo menos oito variantes foram determinadas e nomeadas de acordo com a localização geográfica em que foram originalmente isoladas: *Alaskan*, *China 1*, *China 2*, *Mediterranean* + (*Med* +), *Mediterranean* – (*Med* -), *North Carolina*(60), *Cao*(61) e *C15-NPC*(62) (Figura 1B). Recentemente, descobriu-se uma variante denominada M81, com maior tropismo por células epiteliais que o protótipo B95.8, com múltiplos polimorfismos, que a diferem das outras variantes estudadas, favorecendo esse tropismo celular diferente e a preferência pela replicação lítica(63).

De acordo com mudanças em aminoácidos de LMP1, tanto na região amino-terminal quanto na carboxi-terminal, foram estabelecidos padrões de sequência para as oito variantes mencionadas. Conforme indicado no Quadro 2, cada variante é discriminada pela presença de alterações silenciosas, alterações significativas e alterações únicas de aminoácidos.

As diferenças encontradas entre as variantes de LMP1 aparentemente não alteram seu potencial transformador *in vitro* e não induzem mudanças fenotípicas peculiares em células epiteliais e em linfócitos B. Além disso, a motilidade *in vitro* das células epiteliais humanas induzida por LMP1 é semelhante em todas as variantes avaliadas. Adicionalmente, todas as variantes ativam as vias fosfatidilinusitol-3-quinase (PI3K-Akt) e NF-κB, promovendo efeitos equivalentes no crescimento e transformação celular(60). De maneira semelhante à LMP1 protótipo (B95.8), as variantes interferem com a atividade de uma variedade de proteínas reguladoras do ciclo celular, tais como inibidor de diferenciação 1 (Id1), o inibidor de diferenciação 3 (Id3), a proteína do retinoblastoma (pRb), o inibidor de quinase dependente de ciclina 1 (CDK1 $p27^{Kip1}$), e o inibidor de quinase dependente de ciclina 2 (CDK2)(64,65). Assim, a despeito de alterações de aminoácidos entre as diferentes variantes, muitas propriedades biológicas de LMP1 do EBV não se alteram(60).

Ainda que, aparentemente, as variantes de LMP1 estudadas não apresentem diferenças quanto ao potencial transformador *in vitro* e não induzam fenótipos distintos em células epiteliais e linfócitos B, é possível que tais variantes contribuam para determinar a capacidade de migração e invasão, uma característica importante do

processo de carcinogênese induzida pelo EBV.

Quadro 2: Características das variantes da proteína latente de membrana 1 (LMP1) do vírus Epstein-Barr. (3-366): posição do aminoácido nas sequências das variantes de LMP1.

Variante de LMP1	***Alaskan***	***China 1***	***China 2***	***Med+ e Med-***	***NC***	***C15-NPC***	***CaO***(61)
Alterações significativas região amino-terminal(66)	H-R (3); R-P (13); R-L (17); S-P (22); L-I (25); V-I (43); D-N (46); S-A (57); I-V (63); D-H (67); A-G (82); C-G (84); I-L (85)	H-R (3); R-P (13); R-L (17); D-N (46); D-H (67); A-G (82); C-G(84); I-L (85)	H-R (3); R-P (13); R-L (17) V-I (43); D-N (46); S-A (57); D-H (67); A-G (82); C-G (84); I-L (85)	D-N (46) e I-L (85)	R-L (17); G-R ou G-Q (18); L-I (25); G-I (26); D-N (46); D-H (67); C-G (84); I-L (85)	-	20 aa iniciais com alterações
Alterações N-terminais características(54)(53)(52)(51)	S-P(22) e I-V (63)	L-I(25)	M (25) e I (33);	D-N (46) e I-L (85)	I (37) e T (43)	-	-
Alterações C-terminais características(61)	A (232); N (312); A (313); K (322); A (331); S (345); D (354); V (355)	G-S (212); S-N (309); Q-R (34); L-S (338); S-T (366); Pode haver deleção aa 343 e 352	H (245); D (252 e 344); G-S (212); S-N (309); G-Q (331); L-P (338); G-A ou G-T (355); S-T (366)	S-T (229); S-N(309); Q-R (334); L-S (338); H-R ou deletado (352); S-N ou S-T ou S-A (366)	Q (306); P (313); T (322); N (353); P (358); S-N (309); G-Q (331); L-P (338)	S-T (229); S-N (309); R-Q (322); Q-?(334); L-S (338); S-A (366); Deleção aa 276-280 e 343-352 Inserção aa 298-299	Inserção de 3 sequências repetitivas adicionais de 33pb e ausência dos aa HDPLP (276-285)
Domínio C-terminal PXQXT (região CTAR1 - aa 204 a 208)(61)	-	-	-	-	-	-	-
Domínio C-terminal PXQXS (região CTAR2 – aa 379 a 383)(61)	-	-	-	-	-	-	-
Domínio C-terminal PXQXT (região fora de CTAR1 e CTAR2 -aa 320 a 324) (61)	Lisina (322)	aa 322	-	aa 322	aa 322	-	-
Domínio C-terminal PHDPLP (ligante de JAK3 – aa 302 a 307)(61)	Prolina (306)	-	-	-	aa 306	-	-
Epítopo YLLEMLWRL restrito ao HLA-A2 na região transmembrana (aa 125 a 133)(67)	Y**F**LEI**L**WRL	Y**F**LEI**L**WRL	Y**F**L**DI**LWRL	YLLEI**L**WRL	Não possui essa região	YLL**DI**LWRL	Y**F**LEI**L**WRL
Epítopo YLQQNWWTL restrito ao HLA-A2 na região transmembrana (aa 159 a 167)(62)	-	-	-	-	Não possui essa região	-	-

1.3. LMP1 do EBV na progressão tumoral

A análise de eventuais peculiaridades de variantes de LMP1 em relação ao potencial de disseminação de neoplasias malignas associadas ao EBV é particularmente relevante no carcinoma de nasofaringe (NPC*)*, doença que caracteristicamente apresenta elevada agressividade biológica, com grande tendência a metástases em linfonodos. Células neoplásicas do NPC apresentam programa de latência II do ciclo biológico do EBV, com expressão da proteína LMP1(68).

O surgimento de metástases de um câncer dá-se essencialmente pela colonização de tecidos e órgãos descontínuos em relação ao tumor primário por células neoplásicas malignas, disseminadas por via vascular. O processo é complexo, usualmente dividido em etapas que compõe a chamada "cascata metastática": invasão do tecido adjacente(69), entrada na microvasculatura do sangue ("intravasão")(70), sobrevivência e transporte na corrente sanguínea, aprisionamento em microvasos de tecidos distantes, saída da corrente sanguínea ("extravasão")(71), estabelecimento em tecidos distantes e sua colonização, com formação de tumor secundário macroscópico(72). Há diferentes hipóteses para explicar a aquisição do fenótipo metastático, mas admite-se que células malignas se tornam mais propensas à disseminação, à medida que adquirirem determinadas propriedades biológicas vantajosas, tais como produção de fatores autócrinos de motilidade, aumento da capacidade migratória, capacidade de remodelamento de matriz extracelular, alterações de composição de membrana plasmática etc.(73).

Sugere-se que o favorecimento de disseminação metastática pela LMP1 do EBV no NPC está baseado na ação dessa oncoproteína viral nos fenômenos de degradação da matriz extracelular, modificações na adesão intercelular e/ou supraregulação da motilidade celular e da angiogênese(74).

Há uma clara conexão entre a expressão de metaloproteinases de matriz (MMP), degradação da matriz extracelular, migração e invasão celular(73). A MMP-1 é supraregulada em biópsias de carcinoma de nasofaringe, sendo predominantemente expressa pelas células neoplásicas epiteliais desses tumores. LMP1 propicia não somente a secreção de MMP-1 (75), mas também MMP-9 (76) e MMP-3 (77). Por outro lado, sabe-se que a produção e secreção de MMPs são processos regulados

por componentes da via ERK-MAPK, que também regula a motilidade e invasividade de células epiteliais via CTAR1 de LMP1(78).

Outro fenômeno importante para o potencial migratório e invasor durante a progressão tumoral é a transição epitelial-mesenquimal, na qual células epiteliais adquirem propriedades de células mesenquimais, incluindo maior motilidade e capacidade de remodelar a matriz extracelular (79). A transição epitélio-mesenquimal pode ser induzida por LMP1 por meio da estimulação do fator de transcrição Twist, o que pode ter contribuição da ativação de NF-κB pelas regiões CTAR1 ou CTAR2 da proteína (80).

A proteína LMP1 do EBV pode ser transportada via exossomos, que transferem fatores invasivos e metastáticos para células não infectadas. Essa oncoproteína aumenta a secreção do fator transcricional pró-metastático 1α (HIF1-α) por exocitose. Exossomos $LMP1^+$ e HIF-1α podem balancear os níveis de E- e N-caderina, consistentes com as mudanças associadas à EMT. Células de nasofaringe expostas a exossomos LMP1 positivos com altos níveis de HIF1-α, tendem a aumentar a motilidade celular e o potencial invasivo. Assim, exossomos LMP1 positivos contendo HIF1-α promovem a progressão do NPC por aumentar seu potencial invasor(81).

Por sua vez, a expressão de outro fator de transcrição durante a EMT, Ets-1, estimula a motilidade celular e expressão de ativador de plasminogênio tipo uroquinase (uPA), que se correlaciona com a degradação de componentes da matriz extracelular e facilitação da disseminação metastática (82). A ativação de Ets-1 por LMP1 em células do NPC propicia produção do receptor para o fator de crescimento dos hepatócitos (HGFr), produto do protoncogene c-Met, que está associado à progressão tumoral e metástase em uma variedade de carcinomas (83). A proteína LMP1 pode, ainda, estimular a migração celular e invasividade por meio da supraregulação do receptor Decoy 3 (DcR3), que também causa supressão da apoptose e indução de angiogênese. LMP1 ativa DcR3 por meio da ativação das vias NF-κB e PI3K (84).

O aumento da microvasculatura oferece oportunidade de entrada de células neoplásicas na corrente sanguínea, resultando em aumento de sua distribuição nos tecidos e ocorrência de metástases(74). Hiperatividade do fator de crescimento do endotélio vascular (VEGF), um importante indutor de angiogênese, correlaciona-se com metástases em linfonodos no NPC(85). Sua produção pode ser induzida pela

expressão de COX-2 mediada pela ação de LMP1 na ativação de NF-κB (86), o qual também propicia supraregulação do promotor do gene que codifica IL-8, outra importante molécula pró-angiogênica (87).

Endocan ou molécula 1 específica de células endoteliais (ESM-1) é secretada em resposta a citocinas pró-inflamatórias e fatores angiogênicos como TNF-α, FGF-2 e VEGF. Endocan é um dos principais genes suprregulados por LMP1, em células epiteliais RHEK-1, por meio de vias ativadas pelos domínios CTAR1 e CTAR2, tais como NF-κB, MEK-ERK e JNK. É também supraregulada na linhagem celular de NPC - NPC-TW04. Através dessas vias de sinalização, o Endocan é capaz de estimular a migração e invasão de células endoteliais(88).

A proteína 2 induzida pelo fator α de necrose tumoral (TNFAIP2) é hiperexpressa nos tecidos de NPC e está relacionada a metástase tumoral, contribuindo para a progressão do tumor. A expressão de TNFAIP2 é induzida por LMP1 através da transcrição ativada por NF-κB; pode associar-se a actina, formando protusões de membrana nas células de NPC, contribuindo, então, para a migração celular mediada por LMP1(89).

Um outro oncogene envolvido na indução da proliferação, progressão do ciclo celular, migração e invasão é o fator de iniciação da translação eucariótica 4E (eIF4E). Contribui para a ocorrência e desenvolvimento de malignidades após indução por LMP1 de c-Myc em NPC(90).

Os microRNAs de interferência (miRNA) são fatores que também impactam no desenvolvimento e progressão de neoplasias. Alguns deles são conhecidos como onco-miRNA ou miRNA supressores de tumor. Um exemplo é o miR-155, cuja supraregulação está associada ao acúmulo plasmático de LMP1 durante a infecção pelo EBV em células de NPC. miR-155 induz a proliferação, migração e invasão *in vitro* das células CNE-2 e HONE-1, confirmando seu papel na progressão tumoral do NPC(91).

Dados prévios da literatura sustentam o papel de LMP1 do EBV em fenômenos importantes da carcinogênese, como progressão tumoral, evasão imune e disseminação metastática por indução de migração e invasão celular. No entanto, não existem dados acerca de possíveis diferenças nesses fenômenos que possam ser atribuídas a uma ou mais variantes de LMP1. Em vista do exposto, o presente trabalho foi desenvolvido para a testar a hipótese de que a capacidade de invasão e migração

celular, bem como a expressão de moléculas envolvidas no reconhecimento imunológico das células transformadas, são influenciadas por variações da oncoproteína LMP1 do EBV, o que deve repercutir nos efeitos da infecção pelo EBV na progressão e disseminação de cânceres associados ao vírus.

2. Objetivos

O objetivo do trabalho foi verificar se a transfecção de células HEK293T e NP69, com diferentes variantes de LMP1, altera sua imunogenicidade e confere habilidade distinta de migração e invasão celular.

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Capítulo II

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In vitro migration and cellular invasion of human cells expressing variants of the Epstein-Barr virus LMP1 oncoprotein

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Abstract

The Epstein-Barr virus (EBV) latently infects more than 90% of human adults. Viral infection is associated to the development of some cancers, including nasopharyngeal carcinoma. Oncogenic potential of the virus is usually studied in terms of its capacity to transform the infected cells nevertheless some studies suggest that the EBV infection may also contribute to immune evasion, and cancer progression. Several latent membrane protein 1 (LMP1) variants are described, discriminated mainly by variations in its C-terminal and the transmembrane domains of the protein. LMP1 C-terminal domain activate intracellular signaling pathways that regulated cell migration; moreover, LMP1 upregulate the expression of cell proteins with roles in extracellular matrix remodeling and angiogenesis. Currently it is unknown whether different LMP1 variants possess distinct properties regarding biological phenomena relevant for immune evasion, and cancer progression. Thus, in the present study we evaluated the *in vitro* migration and invasiveness, and immunomodulation of HLA-ABC, HLA-DR, CD80, CD83, CD54, CD40, and PD-L1 of HEK293T cells, and NP69 cell. We observed that Alaskan, Med+, China 1, and China 2 stimulates distinctly migration of NP69 when compared to cell without LMP1. We also saw that HEK293T-China 2 had a pronounced cellular invasion when compared to HEK293T. In regard of expression of molecules involved in immune response modulation, we observed that LMP1 enhanced expression of CD80, and CD83 in NP69-Alaskan cells; and CD54, and PD-L1 in a similar level in NP69-LMP1 cells. In summary, LMP1 promote distinct cellular migration in NP69LMP1 cells, and HEK293T-China 2 invasion, and is capable of modulate molecules involved in immune evasion.

Keywords: EBV, LMP1, NPC, Cellular migration, and cellular invasion.

Introduction

Epstein-Barr virus latently infects more than 90% of human adults. In most cases, primary infection occur between childhood and adolescence[1]. EBV infection is associated with the development of Infectious Mononucleosis (IM) [2] and a variety of human malignancies, including Burkitt's lymphoma (BL), the undifferentiated form of nasopharyngeal carcinoma (NPC)[3], a subset of classical Hodgkin lymphomas (HL), and lymphoproliferative disorders in immunosuppressed patients[4]. These quite different neoplasic diseases share at least one biological aspect related to EBV infection: expression of the viral LMP1 oncoprotein.

LMP1 is the major transforming EBV product. It affects the differentiation of epithelial cells[5,6], and activates many intracellular signaling pathways, notably NF-κB[7,8], Also activates important signal transduction molecules, including the *activator protein 1 (AP-1)*[9], c-Jun N-terminal kinase (JNK)[10], phosphatidylinositol 3-kinase (PI3K-Akt)[11] and STAT[12,13]. The reported effects of LMP1 expression include the upregulation of matrix metalloproteinases 1 (MMP-1) and 9 (MMP-9), notably in malignant cells[14,15], the epidermal growth factor receptor (EGFR)[16], and vascular endothelial growth factor (VEGF)[17], besides induction of anti-apoptotic proteins such as A20, bcl-2 and Mcl1 [18,19].

In addition to this signaling pathways activation, LMP1 has a key role in immunomodulation of molecules involved in T lymphocytes activation and antigen presentation, leading to immune evasion. In fact, LMP1 upregulates HLA-ABC molecules in NPC cells, and cells with LMP1 variants show specific changes in HLA-linked LMP1 epitopes that avoid recognition by cytotoxic T cells.[20].

LMP1, upregulates costimulatory molecule CD83 through NF-κB activation via CTAR1[21]. RelB translocation to the nucleus via LMP1 CTAR1 promotes induction of another costimulatory moleculeCD80[22], while CD54 (ICAM-1) expression can be upregulated by LMP1 in NPC[23], LMP1 mimics CD40 intracellular signaling pathways activation such as NF-κB, which lead to cell survival and EBV infected cells proliferation. In spite of mimics this molecule, LMP1 is also capable of upregulated it expression in NPC cells[24,25].

Finally, LMP1 participate in upregulation of PD-L1 expression through activation of intracellular signaling pathways, such as JAK/STAT, AP-1 e NF-κB[26].

Distinct LMP1 amino acid sequences have been reported, allowing the discrimination of several LMP1 variants[27]. Eight variants were determined and nominated

according to the geographic localization in where they were originally isolated, ***Alaskan***, ***China 1***, ***China 2***, ***Mediterranean*** + (***Med*** +), ***Mediterranean*** - (***Med*** -), ***North Carolina***[28], ***Cao***[29] and ***C15-NPC***[30] (Figure 1B). Even though it was reported that different LMP1 variants apparently do not differ in their *in vitro* transforming potential neither induce sharp distinct phenotypes in epithelial cells and B lymphocytes, it is possible that these variants contributed to determine cellular migration, and invasion, an important characteristic in EBV-induced carcinogenesis.

The malignant cells in NPC show EBV latency type II, usually with LMP1 expression[31], and eventual singularities of their variants regarding its effects in cancer progression may be of special interest in this highly aggressive epithelial cancer, prone to dissemination to regional lymph nodes.

It is suggested that the metastatic spread of EBV-infected malignant cells may be a consequence of extracellular matrix remodeling (ECM), changes in the intercellular adhesion properties, and stimulation of cellular motility and angiogenesis[32], induced by LMP1.

There is convincing data that LMP1 affect cellular motility and lead to higher invasiveness by mechanisms implicating its C-terminal domains CTAR1, CTAR2, and CTAR3[33]. However, it is not known whether these phenomena relevant to immune evasion, and tumor progression could be distinctly induced by LMP1 variants. Therefore, in the present study we evaluated the LMP1 immunomodulatory effect analyzing the expression of HLA-ABC, HLA-DR, CD80, CD83, CD54, CD40, and PD-L1, and *in vitro* cellular migration and invasive potential of HEK293T, and NP69 cells transfected with LMP1 variants.

Material and Methods

Cell culture and cellular transfection

Human embrionary kidney cell - HEK293T were grown in DMEM (Thermo Scientific, Waltham, MS, USA) containing 10% FBS and antibiotics. Human immortalized nasopharyngeal epithelial cell - NP69 were a gift from Dr. Nancy Raab-Traub from North Carolina University, Chapel Hill, NC, and were grown in Keratinocyte-SFM containing 5% FBS, 25µg/mL BPE, 0,2ng/mL EGF (Invitrogen, Carlsbad, CA, USA), and antibiotics. Both

cells lines were maintained in atmosphere with 5% CO_2 at 37°C.

HEK293T cells were transfected with LMP1 vectors (pBabe-B95.8, pBabe-Alaskan, pBabe-Med+, pBabe-China1, pBabe-China2) and control vector – pBabe using *ProFection® Mamalian Transfection System – Calcium Phosphate* (Promega, Madison, WI, USA). Eight to 16h post-transfection we replaced the medium to avoid cytotoxicity. Due to significant cytotoxicity, we changed the calcium-phosphate transfection reagent to *Effectene Transfection Reagent* (Qlagen, Valencia, CA, USA) in order to perform NP69 cell line transfection with pBabe-LMP1 vectors. Sixteen to 24h post-transfection we replaced the medium to remove transfection reagents.

pBabe-LMP1 vectors transformation

pBabe-LMP1 vectors were constructed and gently donated by Dr. Nancy Raab-Traub, North Carolina University, Chapel Hill, NC. In order to construct the LMP1 vectors they used a retroviral vector pBabe-Puro (5169pb) in which were inserted LMP1 variants sequences (B95.8, Alaskan, Mediterranean +, China 1, and China 2). pBabe-LMP1 vectors contain multiple cloning sites – MCS; a SV40 promoter; puromycin resistant genes, and ampicillin resistant gene to bacteria. Total size of pBabe-LMP1 vector is 7207pb (pBabe-B95.8), 6469pb (pBabe-Alaskan, and pBabe-China 2), and 6439pb (pBabe-Med+, and pBabe-China 1). For LMP1 vector transformation E. coli competent cells strain DH5α (Invitrogen, Carlsbad, CA, USA) was used. In summary, we added 1 to 10ng of plasmidial DNA to 100µl of competent cells, and incubated for 30min in ice. Following, cells were submitted to heat-shock at 42°C in water bath for 45s, and for 2min at 4°C. Next, we added 0,9 mL of S.O.C. medium at room temperature; cells were submitted to agitation at 225rpm for 1h at 37°C. Finally, we spread 50 or 100µl of cultured bacteria on LB agar plate with 100µg/mL of ampicillin, and incubated overnight at 37°C.

After transformation, we randomly selected bacterial colonies, which were grown in 2mL LB medium with 100µg/mL of ampicillin for 6h at 37°C, 225rpm. Afterwards, we added 100µl of grown cells to 250mL LB medium with 100µg/mL of ampicillin, and incubated overnight at 37°C, 225rpm. Cells were then submitted to plasmidial DNA extraction and purification using PureLink – Hipure Plasmid Filter Maxiprep (Invitrogen, Carlsbad, CA, USA). After eluting DNA, we performed DNA precipitation with isopropanol to concentrate plasmidial DNA in all samples. To this, we added isopropanol to DNA, and centrifuge for 30min at 4°C, 12000xg; DNA pellet was whashed with 70% ethanol, and

centrifuge for 5min at 4°C, 12000xg. At the end, we resupended DNA pellet in 500µl TE buffer (Tris-HCL 10mM, pH 8,0/EDTA 0,1mM).

Protein extraction and western blot analysis

Total protein was extracted 48h post-transfection using RIPA lysis buffer (Tris-HCL 10mM, pH 7,4/NaCl 150mM/EDTA 1mM/Triton X-100 1%/Deoxicolato de sódio 1%/SDS 0,1%). Following, we assessed protein concentration using *Pierce™ BCA Protein Assay* (Thermo Scientific, Waltham, MA, USA).

An average of 20µg of proteins was loaded on Mini-protean® TGX™ Precast gels – 12% (BioRad Laboratories, Hercules, CA, USA), and submitted to electrophoresis with constant voltage, 110V, for 1 to 2h. Following, we transferred the proteins to a 0,45µm polyvinylidene difluoride (PVDF) membrane (Milipore, Billerica, MA, USA) treated with methanol by semi-dry electroblotting using the device Pierce Fast Semi-Dry Blotter (Thermo Scientific, Waltham, MA, USA) with constant voltage, 15V, for 30 min.

LMP1 expression was assessed by western blot analysis. We used primary antibody against hemaglutinin tag (HA probe (Y-11: sc-805); Santa Cruz Biotechnology, Santa Cruz, CA, USA) to detect LMP1, and normalized the data to GAPDH (GAPDH probe – Poly6314; Biolegend, Inc., San Diego, CA, USA). We used anti-rabbit IgG (complete molecule) – peroxidase antibody produced in goat as a secondary antibody (Sigma-Aldrich, St. Louis, MO, USA). Anti-HA was used at the dilution 1:1000 with overnight incubation at 4°C, followed by secondary antibody incubation, 1:2000, for 1h at room temperature. Otherwise, GAPDH was used at 1:10000 with overnight incubation at 4°C, followed by secondary antibody incubation, 1:5000, for 1h at room temperature. We used Pierce ECL Plus Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA) as a chemiluminescent reagent, and the signal detection was done by autoradiography film - HyBlot CL (Denville Scientific Inc., NJ, USA).

RNA extraction and RT-PCR

RNA extraction were done 48h post-transfection using Purification of Total RNA from animal cells – RNeasy kit (Qiagen, Valencia, CA, USA). Following, we assessed RNA concentration using the device NanoDrop (GE Healthcare, Boston, MA, USA).The RNA was then used for RT-PCR. RNA samples were treated with DNAse (Invitrogen, Carlsbad, CA,

USA) – 4µg of RNA, 10x buffer (1x), *DNAse* (4U), and water to a final volume of 20µl; following we incubated the reaction at room temperature for 1h, and to inactivated the DNAse, we added EDTA (0,1 volume) and incubated samples for 15 min at 65°C. Then, we stored the samples on ice. Afterwards, we realized cDNA synthesis using Reverse Transcription System (Promega, Madison, WI, USA), followed by PCR amplification to validate LMP1 expression. RT-PCR conditions are described in table 1.

Table 1: RT-PCR conditions used to amplify EBV LMP1.

Experiment	Reaction components	Reaction condition
Reserve transcriptase	RNA(1,0µg); $MgCl_2$ 25mM(5mM); Reverse transcription 10x buffer(1X); dNTP mixture(1mM); Recombinant RNasin Ribonuclease Inhibitor(0,5U); AMV reverse transcriptase(15U); random primers (0,5µg); Nuclease free-water: to a final volume of 20µl.	RT - 10min; 42°C – 15min; 95°C – 5min; 4°C – 5 min.
Amplificação por PCR	cDNA(0,5µg); dNTP 10mM(0,2mM); Buffer + $MgCl_2$(1mM); LMP1.S - CGTTATGAGTGACTGGACTGGA(1 pmol); LMP1.A - TGAACAGCACAATTCCAAGG (1pmol); GAPDH.F(1pmol); GAPDH.R (1pmol); Taq polimerase(2,5U); Nuclease feee-water: to a final volume of 25µl. Amplicon: LMP1: DNA: 290pb; RNA: 212pb; GAPDH: 110pb.	94°C – 5min (1X); 94°C – 30s, 57°C – 40s, 72°C – 40s (30X); 72°C – 10min (1X); 4°C - ∞.

RT: room temperature.

NF-κB luciferase reporter assay

We performed NF-κB luciferase reporter assay48h post-transfection using Dual Luciferase® Reporter Assay System (Promega, Madison, WI, USA), and vectors to *Firefly*, and *Renilla* luciferase (pNF-κB e pGL4.74[hRLuc/TK]). HEK293T cells (1x10^6 cells) were transfected with pBabe-LMP1 vectors (4μg), pNF-κB (2,5μg), and pGL4.74[hRLuc/TK] (5μg) using ProFection® Mamalian Transfection System – Calcium Phosphate (Promega, Madison, WI, USA). *Firefly*, and *Renilla* luciferase signals were evaluated using the device Luminometer Glomax (Promega, Madison, WI, USA), and we normalized the data to the ratio *Firefly*/luciferase *Renilla* (F/R) accordantly to the manufacturer's instructions(92).

Migration assay

In order to evaluated cellular migration we used scratch wound healing assay, and transwell migration assay. To perform the scratch wound healing assay, HEK293T, and NP69 cell lines were grown to 80-90% confluence in 12 well plates, and transfected with pBabe-LMP1 vectors. Twenty-four hours post-transfection, cellular monolayer, expressing or not LMP1, was damaged with a 200μl micropipette tip, drawing two shelves in the well. We gently removed detached cells by Dulbecco's phosphate buffered saline (DPBS) washes, and added fresh medium (DMEM or Keratinocyte-SFM, depending on the cell used). We performed photomicrography immediately, 8, 12 or 24h afterwards, while incubation at 37°C with 5% de CO_2. We analyzed images using MRI Wound Healing tool from ImageJ(93).

For the transwell migration assay, we used a 24-well transwell permeable support system 8,0μm pores (Corning, Inc., NY, USA). NP69 cell line were grown to 80-90% confluence in 24 well plates, and transfected with pBabe-LMP1. Twenty-four hours post-transfection we plated 5x10^5 cells in serum-free medium in the upper chamber, and the lower chamber were filled with complete medium supplemented with a chemoattractant reagent – epithelial growth factor (EGF) (0,2ng/mL) (Invitrogen, Carlsbad, CA, USA). After incubation for 24h, migrating cells at the lower chamber were counted. The transwell insert was also recovered, washed, and stained with 0,1% toluidine blue for counting migrating cells.

Invasion assay

In order to estimate the cellular invasion ability of transfected cells, we also used a transwell system, working with the HTS transwell® - 96 well plate 8,0µm (Corning, Inc., Corning, NY, USA) and matrigel phenol red free (Corning, Inc., Corning, NY, USA). For stimulating tissue resistance, phenol red-free matrigel (5ng/mL) was added to the transwell insert (50µl), and incubated overnight at 37°C, 5% CO_2. Transfected HEK293T and NP69 cells were plated 48h post-transfection in the upper matrigel coated chamber ($2x10^4$ cells/50µl serum-free medium). In the lower chamber, we added complete medium supplemented with the chemoattractant reagent - EGF (10ng/mL) (Invitrogen, Carlsbad, CA, USA). After 24h, we fixed the invading cells with 3,7% paraformaldehyde for 2 min, permeabilized cells with 100% methanol for 20 min, and stained with Giemsa for 30 min. Then, cells stained were photographed and analyzed using Analyze particles tool from ImageJ(93).

Cell phenotyping

LMP1-transfected NP69 cells were also evaluated regarding modulation of surface molecules. For this, 48h post-transfection with pBabe-LMP1 vectors and pBabe vector (empty vector) we immunophenotyped cells by flow cytometry. Briefly, $1x10^5$ cells were added to FACS tubs, and centrifuge for 30s at 10000rpm, and stained with monoclonal antibodies anti-HLA-ABC-Alexa488, anti-HLA-DR-PE, anti-CD80-APC-H7, anti-CD83-PE-Cy7, anti-CD54-PE, anti-PD-L1-PE, and anti-CD40-PE for 20min at 4°C. After incubation, cells were washed twice with 200µl of cytometry buffer (Isoton 0,5% BSA), and suspended in 300µl of cytometry buffer. Events were acquired using FACSCanto™II cytometer (BD Biosciences), and data analyzed using FlowJo software (Tree Stars Inc.).

Apoptosis and cell death

In order to analyze NP69-LMP1 cell death, $2x10^5$ cells were added to FACS tubs, and centrifuge for 30s at 10000rpm. Cells were stained with Anexin V (for late apoptosis), and 7-aminoactinomycin D (7AAD) (for necrosis) for 20min at room temperature. After incubation, cells were washed twice with 200µl of cytometry buffer, and suspended in

300μl of the same buffer. Events were acquired using FACSCanto™II cytometer (BD Biosciences), and data analyzed using FlowJo software (Tree Stars Inc.).

Statistical analysis

Data are expressed in mean ± SD. One-way ANOVA was performed for statistical comparisons, followed by Tukey's test. We used GrapphPad Prism 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. Statistical significance was assumed at a p value ≤0.05.

Results

LMP1 expression by transfected HEK293T and NP69 cells

The first step of this study was the standardization of transfection method, protein extraction, and western blot using HEK293T cells. Our preliminary assay showed that the antibody anti-LMP1 (anti-human LMP1; BD Pharmigen™) was not able to detect all LMP1 variants (figure 1).

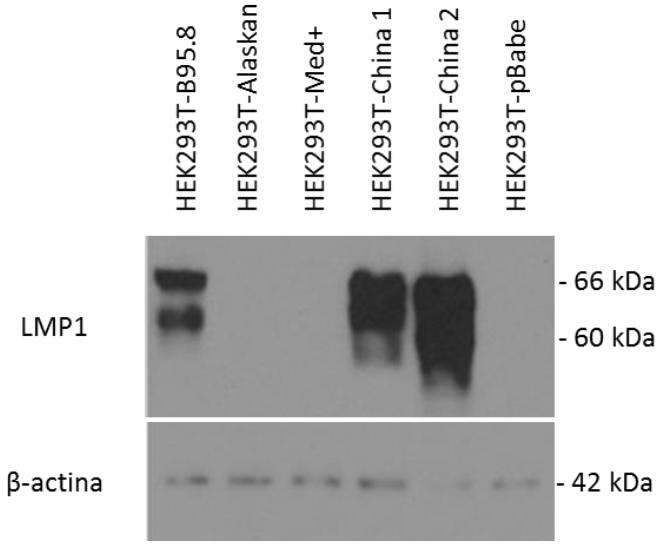

Figure 1: Western Blot performed with anti-LMP1 and β-actina antibodies. We used 20µg of total protein, extracted from HEK293T cells 48h post-transfection with pBabe vectors expressing EBV LMP1 variants. LMP1: 60-66kDa; β-actina: 42kDa.

Considering that pBabe-LMP1 vectors are hemaglutinin-tagged (HA-tag), we used an anti-HA antibody to confirm the successful of transfection. As depicted at figure 2, HA-tagged LMP1 were detected in the lysates of HEK293T expressing LMP1, but not in HEK293T cells transfected with pBabe (empty vector – negative control).

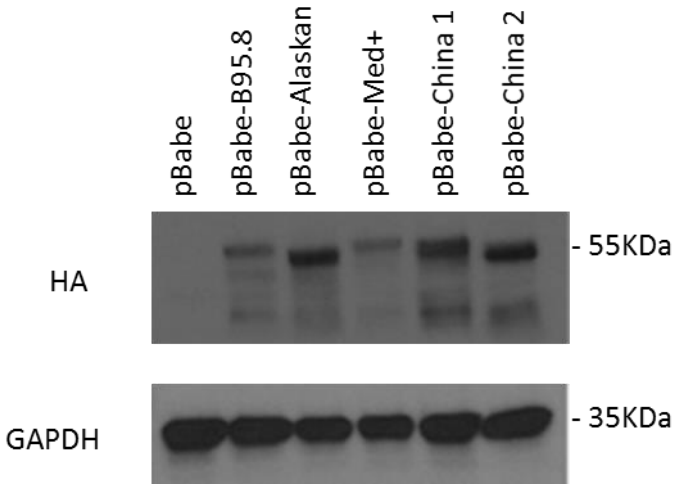

Figure 2: Western Blot performed with anti-HA (EBV LMP1), and GAPDH (control) antibodies. We loaded 20µg of total protein, extracted from HEK293T cells 48h post-transfection with pBabe vectors expressing EBV LMP1 variants. HA-tag LMP1: 50-55kDa; GAPDH (control): 35kDa.

Migration ability of HEK293T cells

Once we confirmed HEK293T and NP69 transfection with all LMP1 variants, these were evaluated for their cell migration capacity.

Figure 3A shows the results of one out of six independent migration assays, representing the scratch wound healing in the HEK293T cell monolayer. It is possible to observe that cells, transfected or not with LMP1, migrate to other side of wound in order to heal the cell monolayer damage during the observation time - 24h, decreasing the damaged area. Figure 3B illustrates the mean of six independent experiments performed in triplicate, and indicates that cell migration capacity do not depend on the LMP1 variant transfected into the cells.

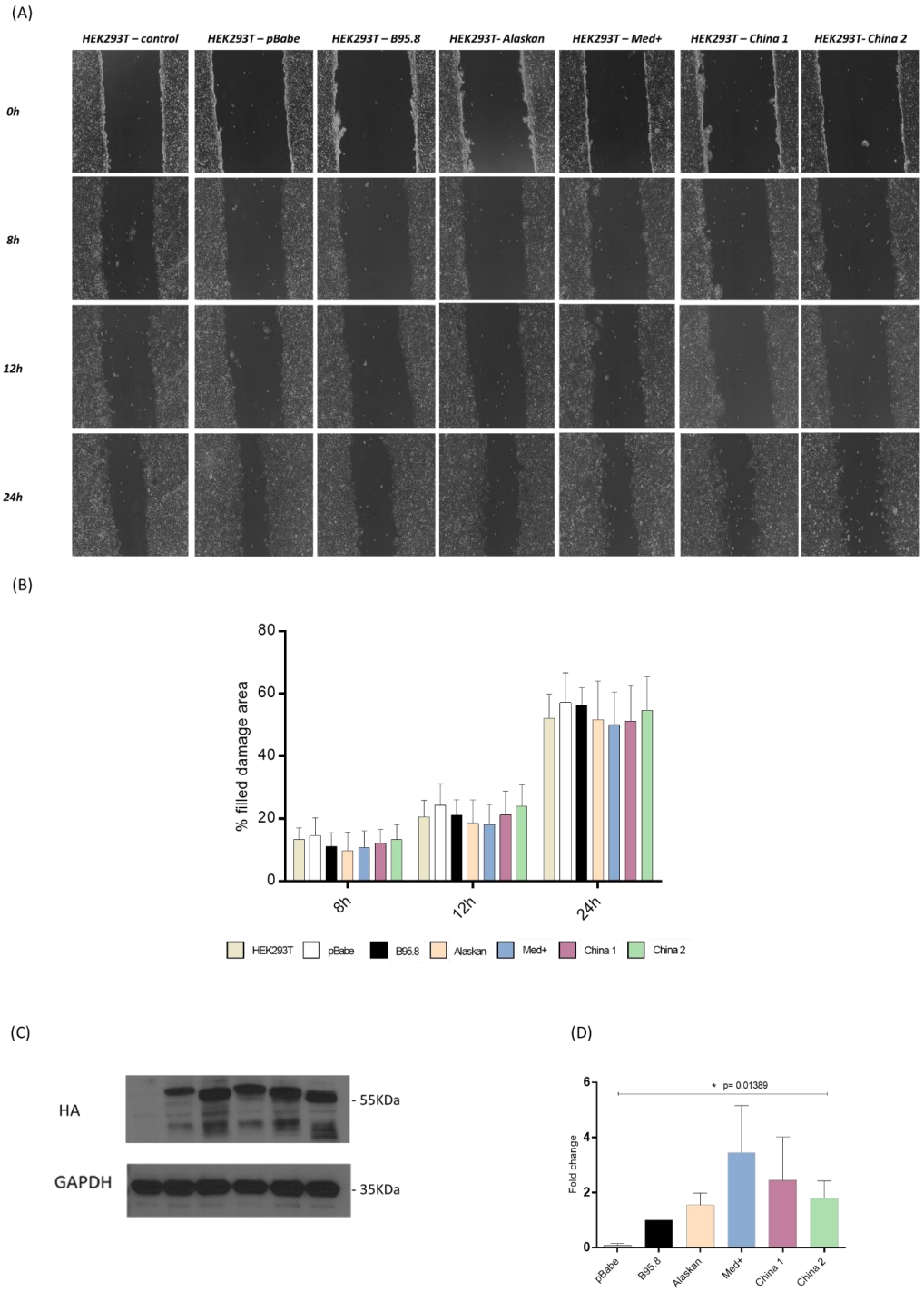

Figure 3: Cell migration assay performed with HEK293T cells, expressing or not LMP1. (A): Filled damage area were quantified at time points 0, 8, 12 e 24h. (B): Graphical analysis of filled damage area (%). (C): LMP1 western blot detection in HEK293T cells expressing EBV LMP1 variants. HA-tagged LMP1: 55kDa; GAPDH (control): 35kDa. (D): Western blot normalization using the ratio HA-tagged LMP1/GAPDH. We performed six independent cell migration assay in triplicate. Statistical significance was assumed as *p≤0.05.

Note that all experiments were validated by western blot for LMP1 detection (figure 3C). One important step in western blot validation is the protein normalization. To this, we calculated the quantification ratio of HA-tagg LMP1/GAPDH. As a result, we have the fold change that indicate how much times one cell express more LMP1 than another. Figure 3D shows that HEK293T-Alaskan (0.54 fold), HEK293T-Med+ (2.4 fold), HEK293T-China 1 (1.4 fold), and HEK293T-China 2 (0.80 fold) express more LMP1 than the prototype B95.8.

Functional activity of LMP1 in transfected cells

One could argue that transfection of HEK293T with p Babe-LMP1 vectors could be able to block LMP1 post-transfection. In order to clarify if transfection could interfere hinder functional activity of these cells, we performed a NF-κB luciferase reporter assay to verify LMP1 activity post-transfection. In this assay, we used the Dual Luciferase® Reporter Assay System (Promega, Madison, WI, USA), and the manufacturer suggested that we use a NF-κB Firefly luciferase vector (pNF-κB) to evaluate LMP1-induced NF-κB activity and a Renilla luciferase vector (pGL4.74[hRLuc/TK]) to normalize the luminescence signal from the firefly vector.

Figure 4 shows a tendency of high NF-κB levels in cells transfected with some LMP1 variants - Alaskan, Med+, China 1, China 2, and low levels in cells transfected with the prototype variant - B95.8. Thereby, we demonstrated that LMP1 activity in HEK293T cells were not inhibited by pBabe-LMP1 vector transfection.

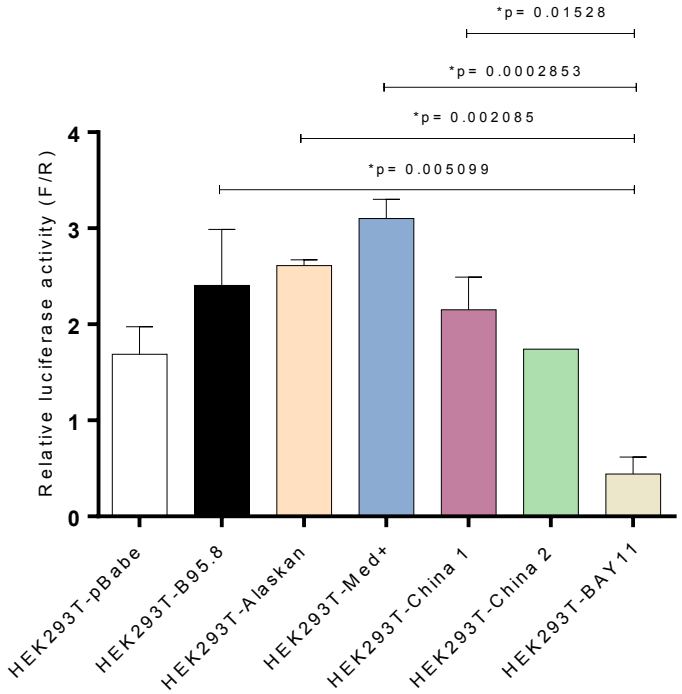

Figure 4: LMP1-induced NF-κB activity. NF-κB luciferase reporter assay to validate LMP1 activity post-transfection. Data were normalized, and presented as the ratio *Firefly* luciferase (F)/*Renilla luciferase* (R) - (F/R). HEK293T-BAY11: cells transfected only with Firefly, and Renilla luciferase vectors, and treated with BAY11, a NF-κB inhibitor. Experiments performed in triplicate. Statistical significance was assumed as *p≤0.05.

Effect of LMP1 transfection on the migration ability of NP69 cells

Since NP69 cells in vitro mimic EBV infection in a better way than HEK293T cells, those cells were also transfected to evaluate the effects of LMP1 transfection.

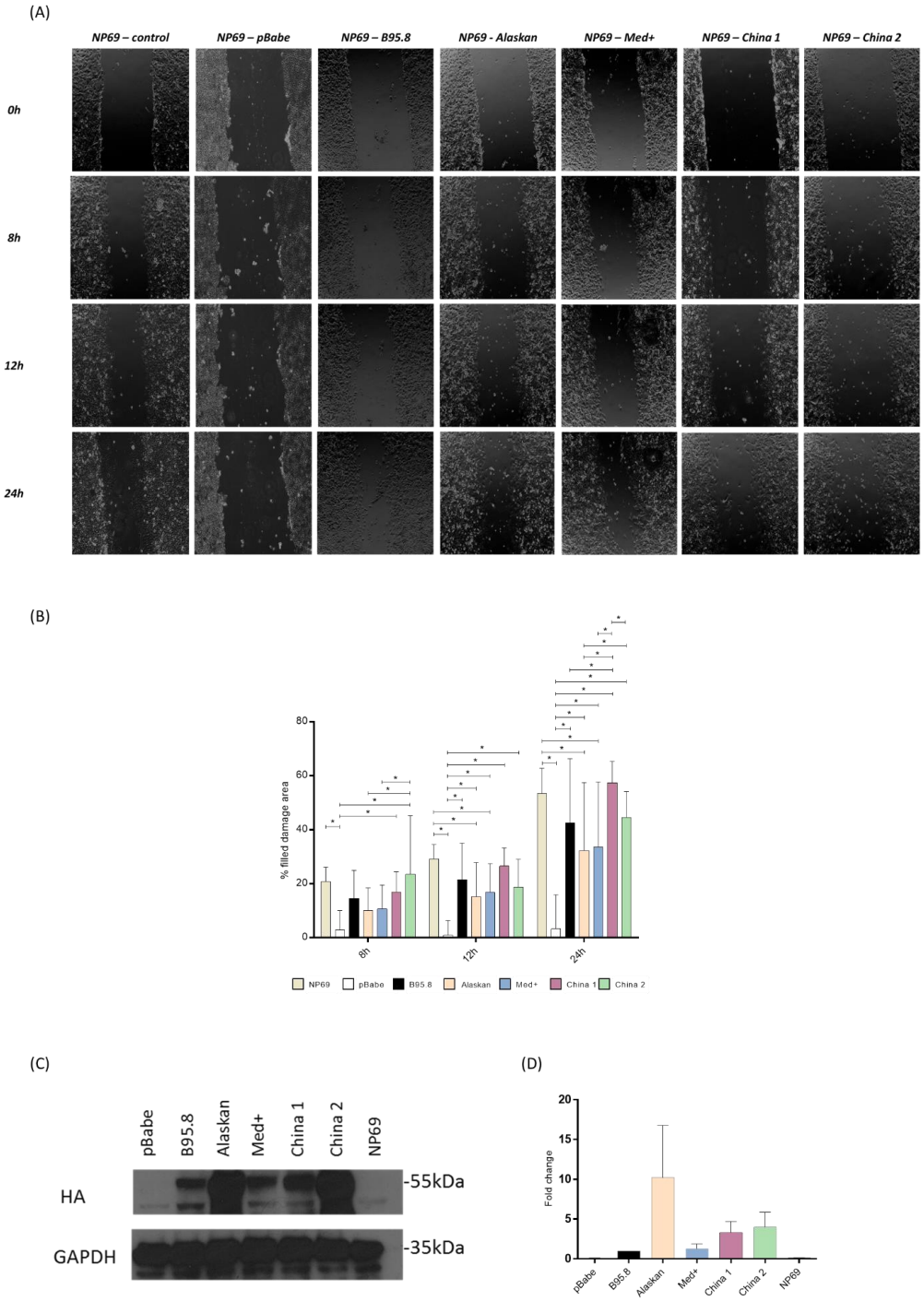

Figure 5: Cell migration assay performed with NP69 cells, expressing or not LMP1. (A): Filled damage area were quantified at time points 0, 8, 12 e 24h. (B): Graphical analysis of filled damage area (%). (C): LMP1 detection by western blot in NP69 cells expressing EBV LMP1 variants. HA-tagged LMP1: 55kDa; GAPDH: 35kDa. (D): western blot normalization using the ratio HA-tagged LMP1/GAPDH. We performed four independent cell migration assay in triplicate. Statistical significance was assumed as *p<0,05.

Figure 5A shows that transfected cells with different LMP1 variants have distinct cell migration patterns both compared among each other and with NP69-pBabe (empty vector), and NP69.

Figure 5B shows the differences of cell migration patterns between NP69-pBabe and NP69-Med+, NP69-pBabe and NP69-China 1, NP69-pBabe and NP69-China 2. We also observed differences between NP69-Alaskan and NP69-China 2, NP69-Alaskan and NP69-China 1, NP69-Med+ and NP69-China 1; NP69-Med+ and NP69-China 2, and NP69-China 1 and NP69-China 2.

Experiment were also validated by western blot (figure 5C), and the index of LMP1 fold change is represented at figure 5D, showing that NP69-Alaskan (9.1 fold), NP69-China 1 (2.2fold), and NP69-China 2 (2.9 fold) express more LMP1 than the variant prototype B95.8.

NP69 chemotaxis migration

In addition to cell migration, we decided to evaluated whether LMP1 variants influences the ability of cells to respond to chemotactic stimulation. For this purpose we analyzed the number of cells that chemotactly migrated to EGF stimulation in a transwell system.

In figure 6 is possible to observe a representative assay of an experiment performed in duplicate.

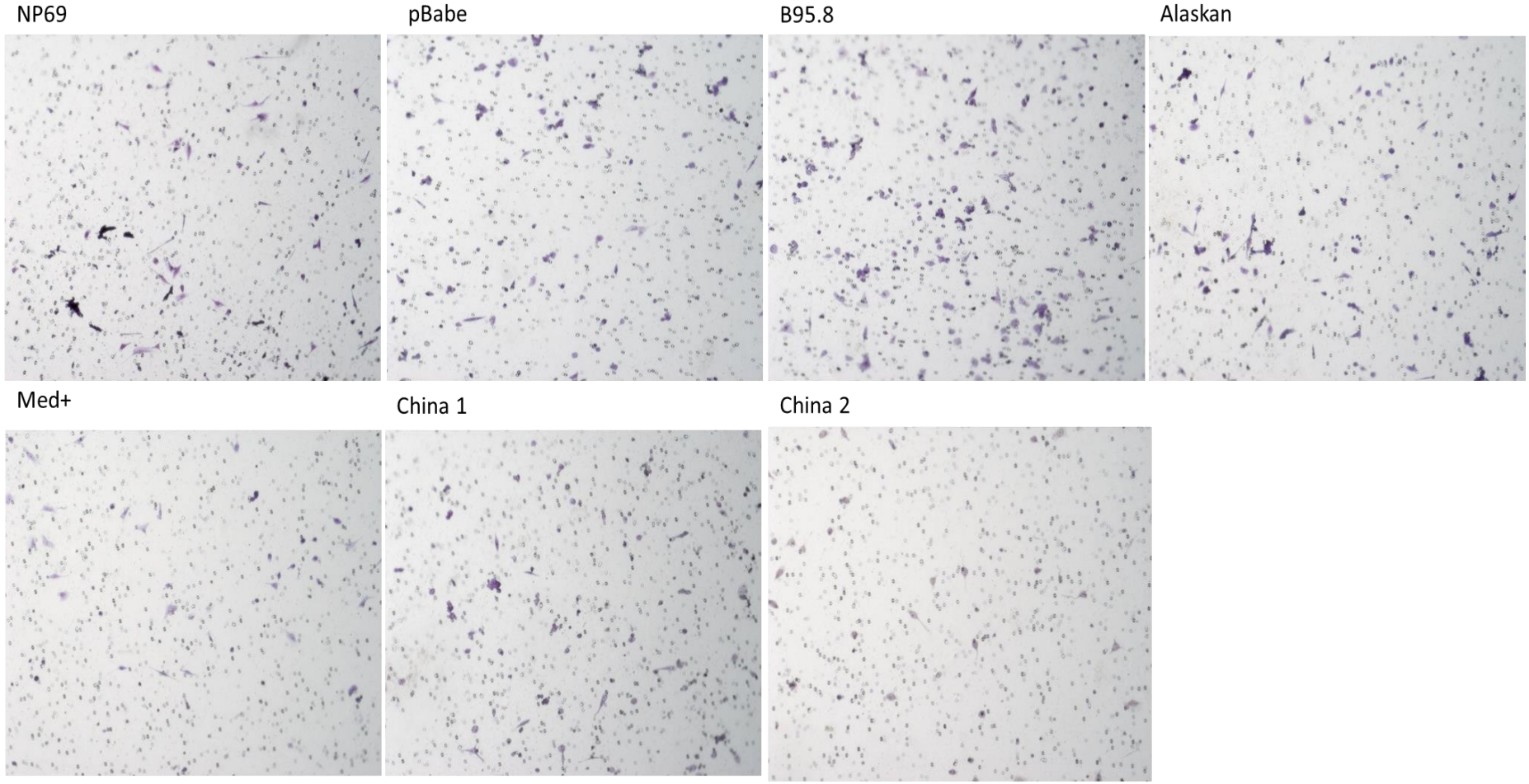

Figure 6: NP69 chemotaxis migration assay. Representative assay of an experiment performed in duplicate. After 24h, migrated cells were fixed, stained with Giemsa, and photographed.

In this assay, we analyzed migrated cells that were in the transwell insert (figure 7A), and in the supernatant of lower chamber (figure 7B). As we can see in the figure, there is no difference in this pattern when compared NP69-LMP1, NP69-pBabe (empty vector), and NP69 cells.

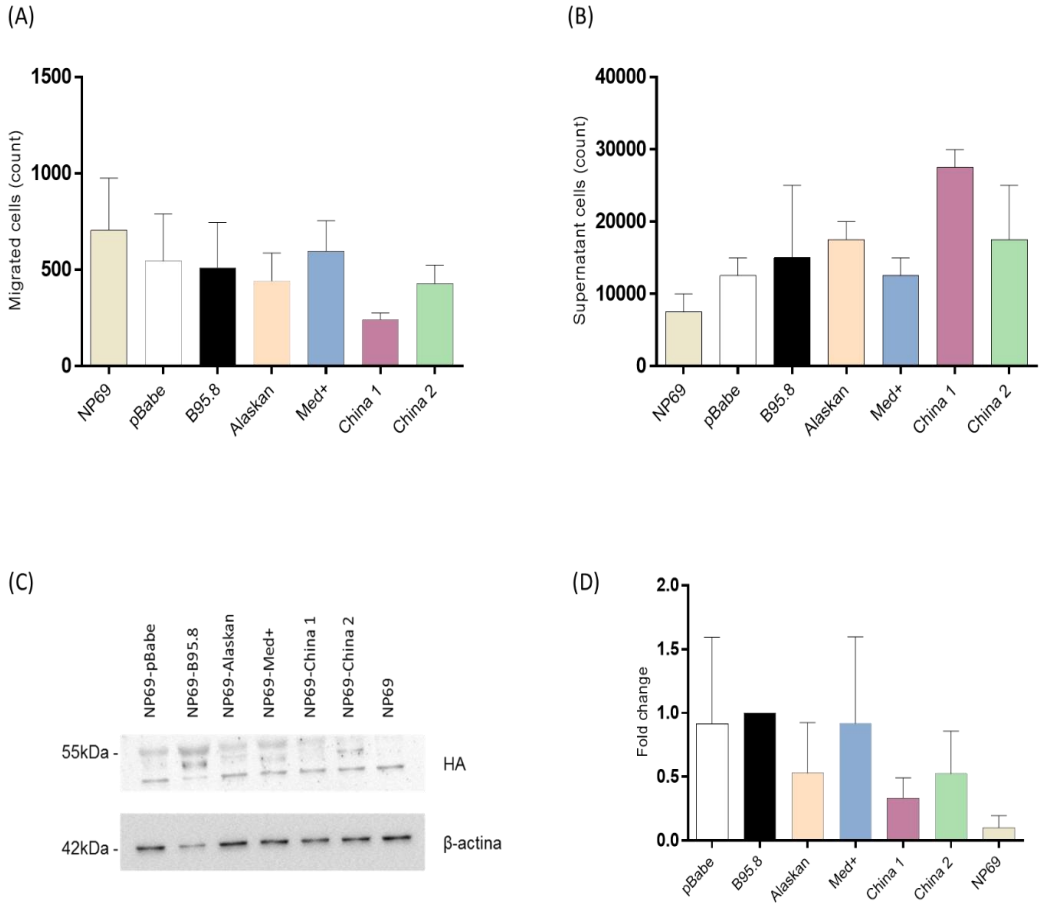

Figure 7: NP69 migration assay in response to the chemoattractant EGF. (A): Graphical analysis of migrated cells in the transwell inserts. (B): Graphical analysis of migrated cells in the supernatant of lower chamber. (C): LMP1 western blot detection. HA-tagged LMP1: 55kDa; β-actina: 42kDa. (D): Normalized western blot data using the ratio HA-tagged LMP1/GAPDH. Statistical significance was assumed as *p<0.05.

This assay was validate by LMP1 western blot detection (figure 7C), and normalized western blot data show that NP69-LMP1 had similar LMP1 levels when compared to NP69-B95.8 (LMP1 prototype) (figure 7D). Therefore, NP69 cells, expressing or not LMP1, respond in a similar way to this chemoattractant stimulus – EGF.

Cell invasion assay was performed with both cells to verify if it is possible to observe differences among cells expressing or not LMP1.

HEK293T and NP69 cell invasion

Since tissue invasion is an important feature of malignant cells, we investigated the effect of LMP1 transfection in this parameter, using a transwell system in which a matrigel layer simulated the tissue resistance.

Transwell chamber was washed 24h later, then invading cells were fixed and stained with Giemsa for microscopic analysis. Cells at transwell membrane were photographed for further image analysis.

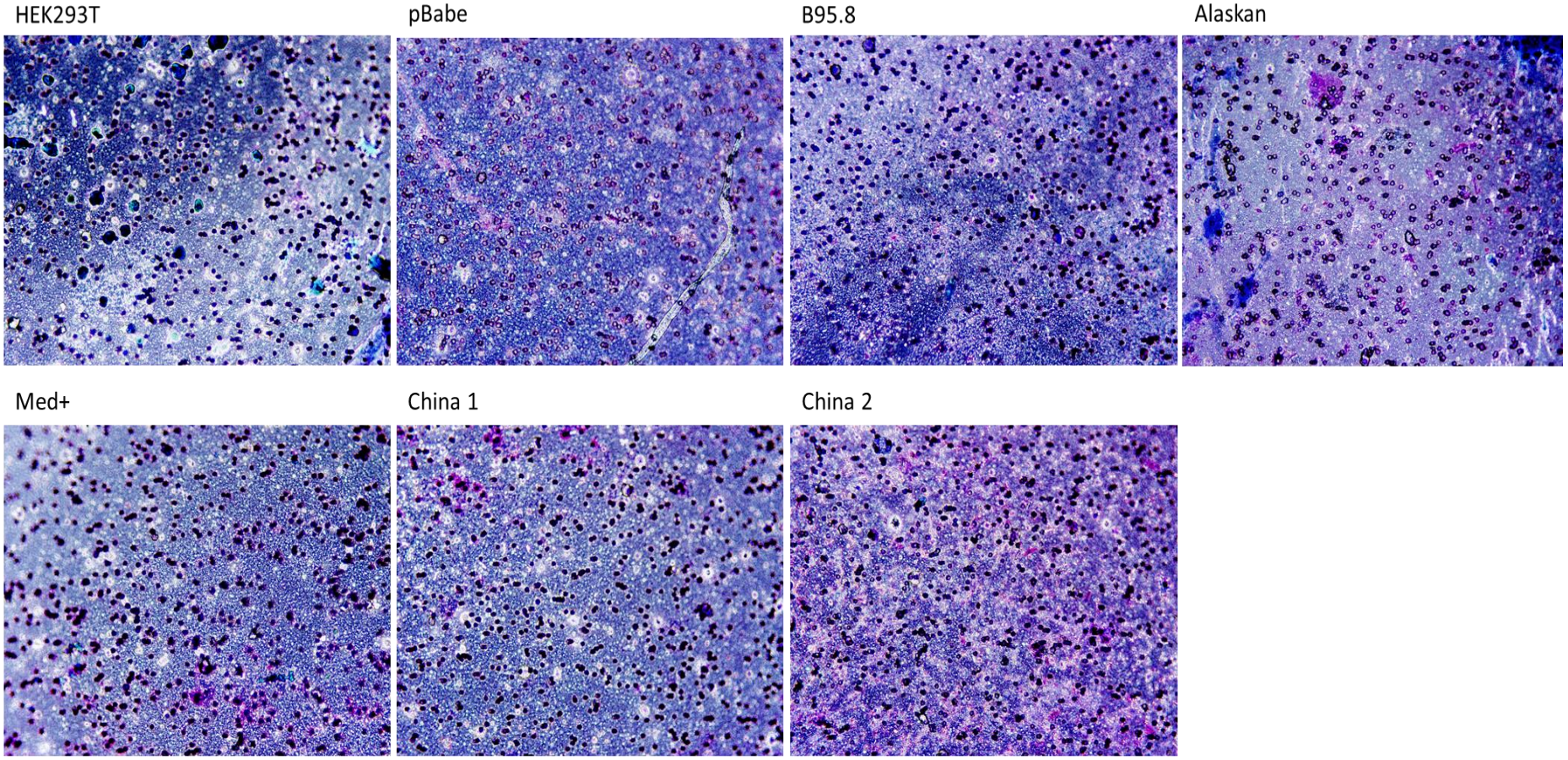

Figure 8: HEK293T cell invasion assay. Representative assay of four independent experiments performed in triplicate. After 24h, invaded cells were fixed, stained with Giemsa, and photographed.

When we analyzed the data generated with HEK293T cells, expressing or not LMP1, we observed that only HEK293T-China 2 shown an enhanced invasion pattern when compared to HEK293T (figure 9A).

HEK293T invasion assay was validated by LMP1 western blot detection (figure 9B). Normalized LMP1 expression shown that HEK293T-China 1 (1.5 fold) and HEK293T-China 2 (3 fold) expressed more LMP1 than the prototype B95.8 (figure 9C).

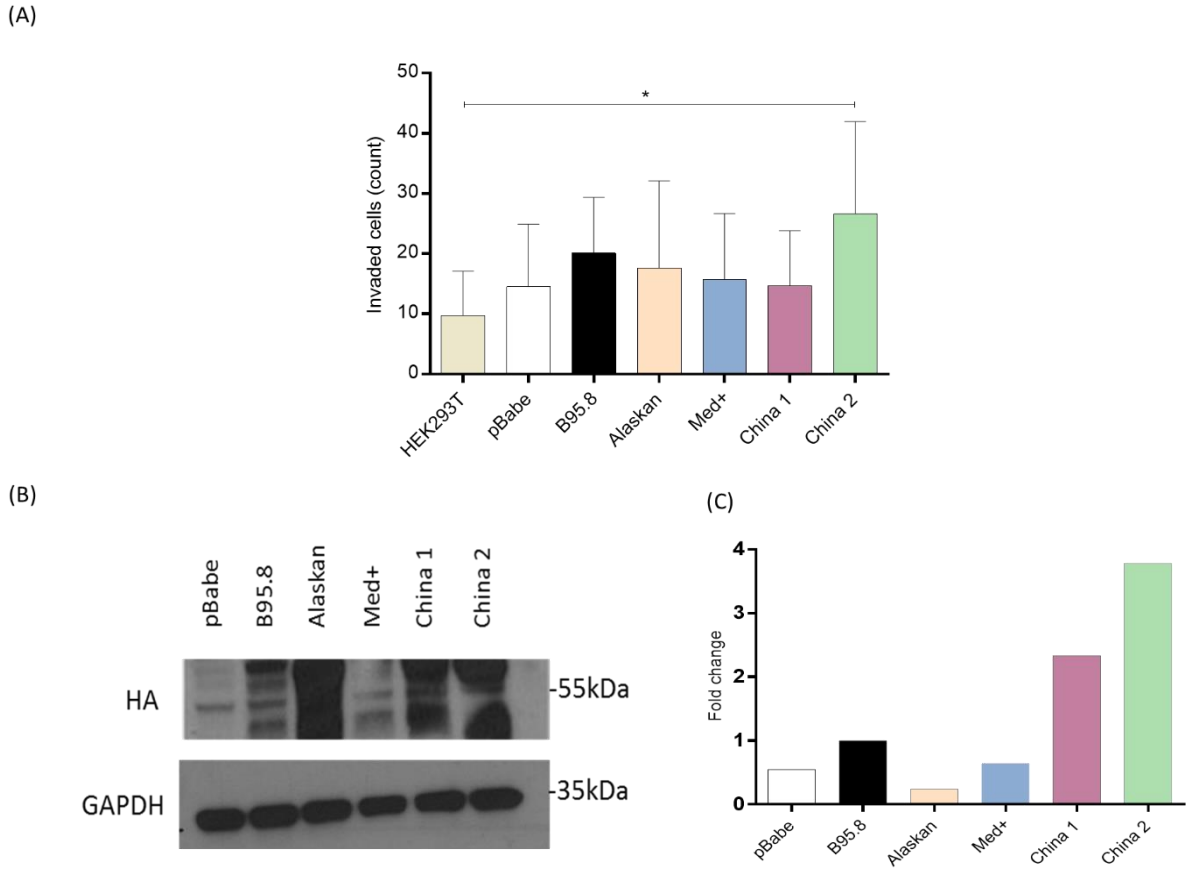

Figure 9: HEK293T cellular invasion assay. (A): Graphical analysis of invaded cells (count of invaded cells). (B) LMP1 western blot detection. HA-tagged LMP1: 55kDa; GAPDH: 35kDa. (C): Western blot normalization using the ratio HA-tagged LMP1/GAPDH. We performed four independent cellular migration assay in triplicate. Statistical significance was assumed as *$p<0.05$.

Based on this results, cell invasion assay with NP69, expressing or not LMP1, was also performed following the same protocol (figure 10).

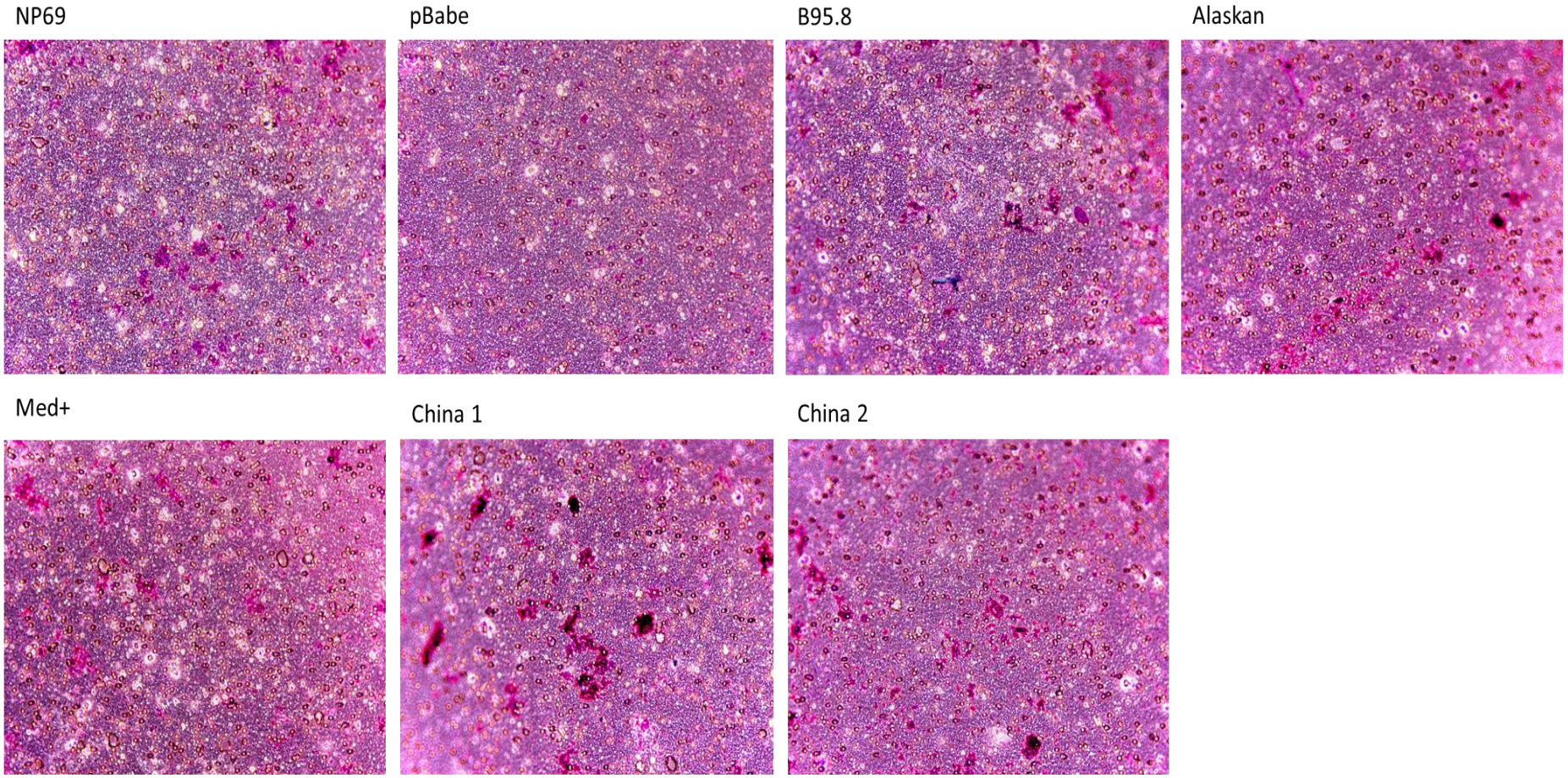

Figure 10: NP69 cellular invasion assay. Representative assay of four independent experiments performed in triplicate. After 24h, invaded cells were fixed, stained with Giemsa, and photographed.

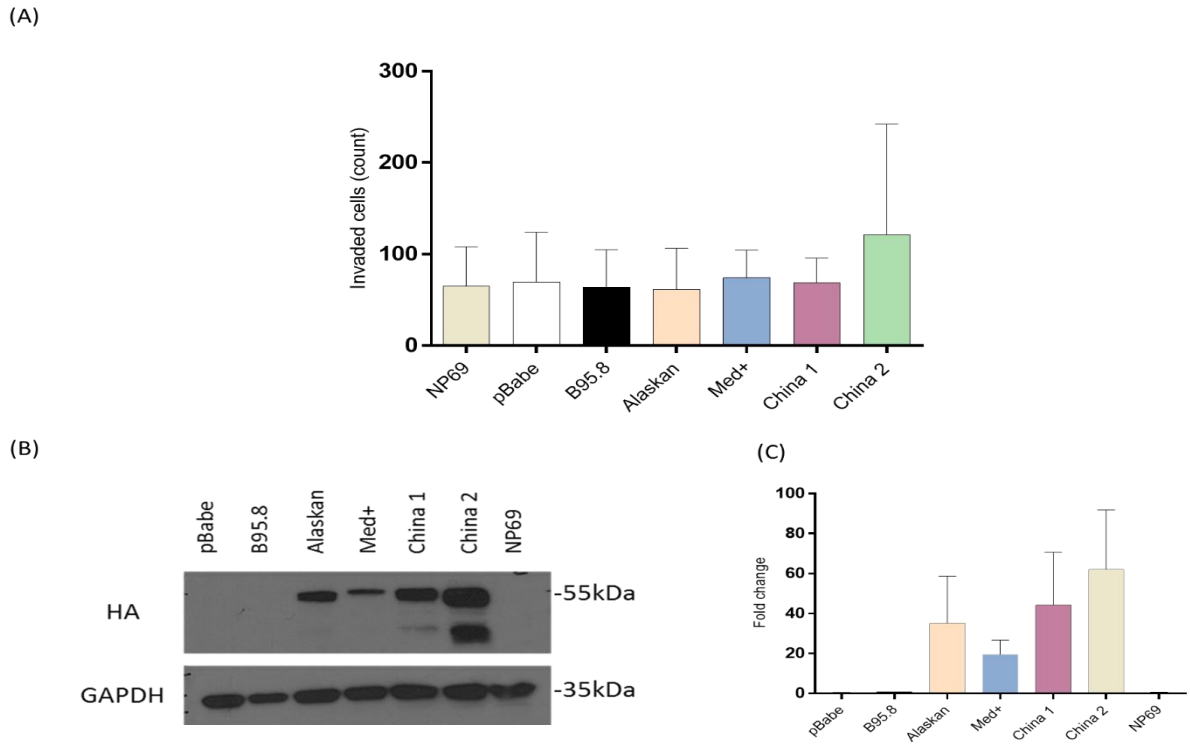

Figure 11: NP69 cellular invasion assay. (A): Graphical analysis of invaded cells (count of invaded cells). (B) LMP1 western blot detection. HA-tagged LMP1: 55kDa; GAPDH: 35kDa. (C): Western blot normalization using the ratio HA-tagged LMP1/GAPDH. We performed four independent cellular migration assay in triplicate. Statistical significance was assumed as *p<0.05.

We can see in Figure 11A, that there are no differences in cell invasion pattern when we compared NP69-LMP1 variants among each other, neither when compared NP69-pBabe and NP69 with NP69-LMP1.

LMP1 western blot expression was seen in the NP69-LMP1 cells, except for the absence of LMP1 in the prototype B95.8 (figure 11B). Surprisingly, normalization of western blot data indicates that differences between LMP1 variants varied from 1.51 fold (NP69-Med+) up to 61.11 fold (NP69-China 2) (figure 11C).

NP69 cells immunophenotype

Since LMP1 can influence the cell expression of membrane molecules involved in both cell adherence and immune modulatory role, we investigated weather LMP1

variants are able to change the expression of some selected markers of transfected cells.

We analyzed HLA-ABC, and HLA-DR – antigens located on major histocompatibility complex, CD80, and CD83 molecules from immunoglobulin superfamily, CD54 – intracellular adhesion molecule upregulated in EBV-associated malignancies; CD40 – transmembrane molecule from necrosis tumoral receptor superfamily, and PD-L1, known as immune suppressor factor, and upregulated in NPC EBV positive.

Figure 12A illustrates the dot plot graphs of NP69, NP69-LMP1, and NP69-pBabe based on cell size and granulosity. We observed that distribution of dots changed when NP69 cells were transfected with pBabe or pBabe-LMP1 vectors, suggesting that cell morphology was influenced by transfection. The transfection was again validated by LMP1 western blot detection (figure 11B). Normalized data, show that NP69-B95.8 cells express more LMP1 than cells containing the other LMP1 variants (figure 11C).

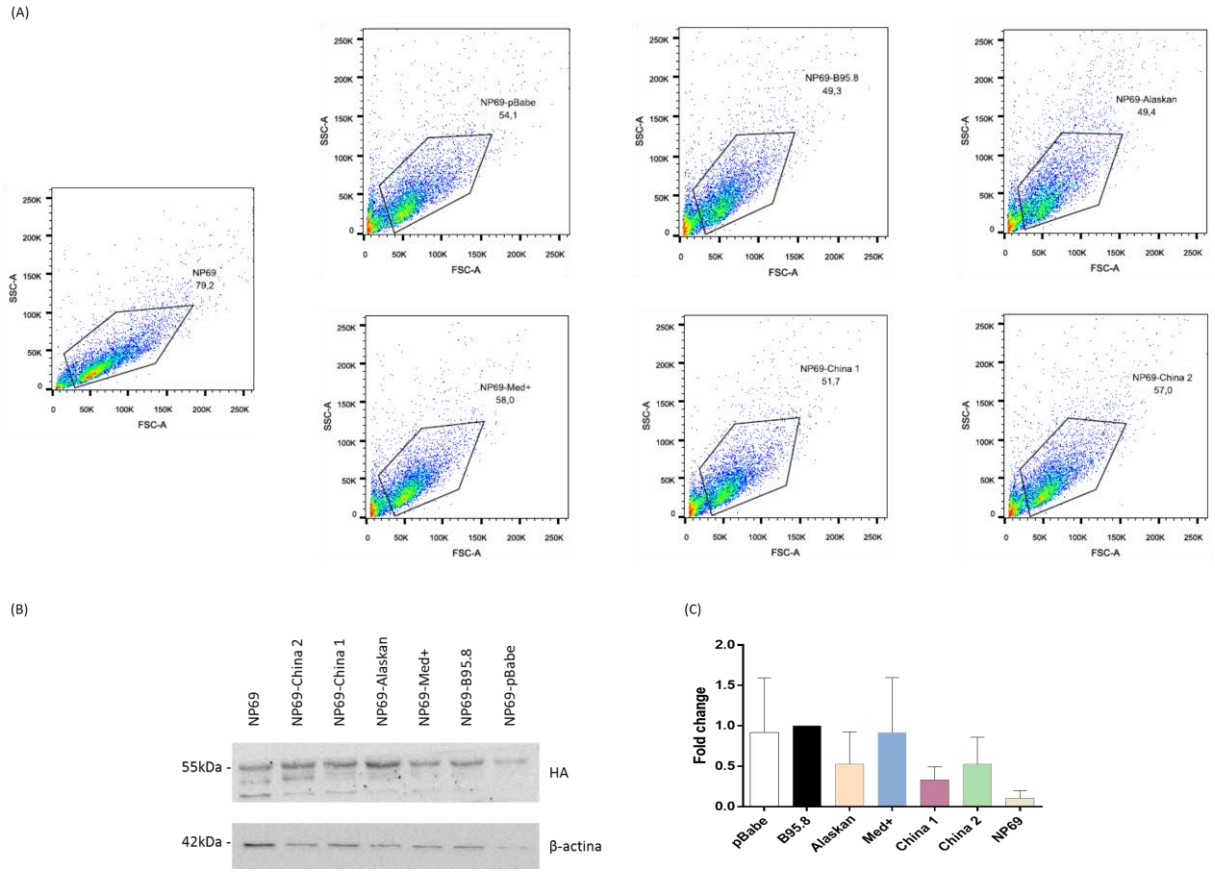

Figure 12: (A): Distribution of based on size and granulosity, after transfection with pBabe (empty vector), and pBabe-LMP1 (B95.8, Alaskan, Med+, China 1 e China 2) vectors. (B) LMP1 western blot detection. (C): LMP1 normalized data. HA-tagged LMP1: 55kDa; β-actina: 42kDa. Independent transfections performed in triplicate to each flow cytometry analysis.

In figure 13 we represent histograms of CD80, CD83, and PD-L1 expression by NP69 cells, and NP69 cells transfected with pBabe vector (empty vector) or pBabe-LMP1 vectors (B95.8, Alaskan, Med+, China 1, and China 2). Details of immunophenotyping are represented at the Table 2.

Table 2: Immunophenotyping of NP69 cells transfected with LMP1.

	Molecules analyzed (%)						
Cells lines	HLA-ABC	HLA-DR	CD80	CD83	CD54	CD40	PD-L1
NP69 (control)	99,7	0,050	1,87	7,13	98,0	1,17	17,0
NP69-pBabe	99,8	0,055	4,61	22,3	98,0	3,95	49,6
NP69-B95.8	99,7	0,061	5,70	28,8	97,4	2,84	43,5
NP69-Alaskan	99,2	0,16	10,6	48,9	95,0	2,52	43,8
NP69-Med+	99,1	0,034	2,12	18,9	97,7	3,62	44,0
NP69-China 1	99,6	0,058	4,45	23,6	97,1	2,41	48,7
NP69-China 2	99,7	0,11	4,24	19,3	97,4	3,70	37,9

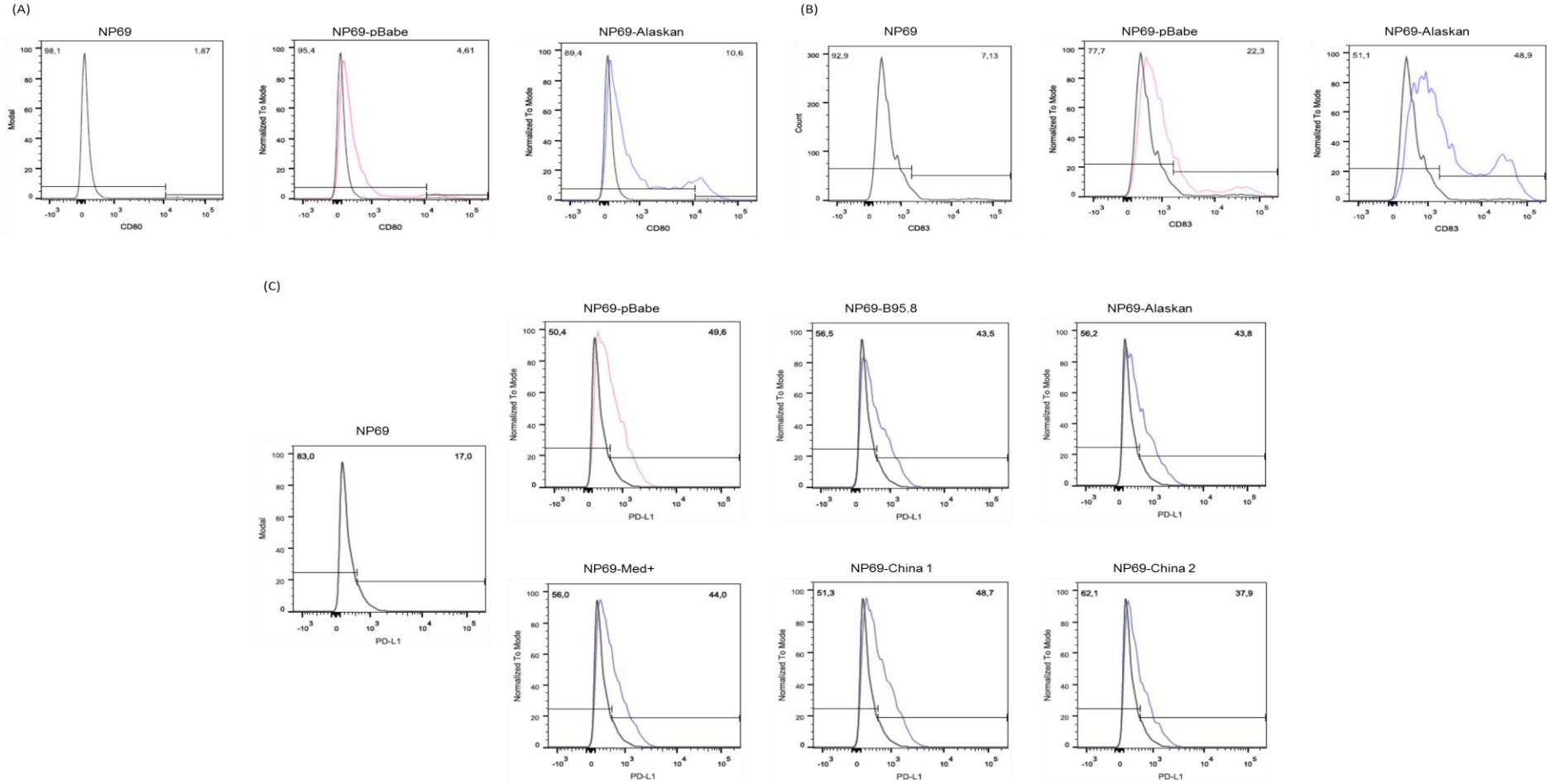

Figure 13: Flow cytometry analysis of NP69 cells. (A): Flow cytometry analysis of CD80. (B): Flow cytometry analysis of CD83. (C): Flow cytometry analysis of PDL-1. NP69, grey line; NP69-pBabe (empty vector), light pink line; NP69-LMP1 (B95.8, Alaskan, Med+, China 1 e China 2), blue line.

Analysis of CD80 and CD83 on NP69 cells showed that 10.6% of cells transfected with Alaskan vector expressed CD80 marker, while just 1.87% of NP69 control cells expressed this marker (figure 13A). Curiously, almost half of cells with Alaskan variant expressed the CD83 marker, and only 7.13% of untransfected cells (control cells) presented it (figure 13B). CD83 expression was also increased in cells transfected with China 1 and China 2 variants although in lower frequency than Alaskan (Table 2).

Similar percentage of both control NP69 and LMP1 transfected NP69 cells expression (expression average of 99%), while less than 1% expressed HLA-DR molecules (Table 2).

We also investigated the expression of CD54, a cell adhesion molecule (also known as ICAM-1). Analysis revealed that almost all NP69 cells express this marker (98%) and transfection with LMP1 vectors just slightly changed the expression of this marker (Table 2). In opposition, just a very small number of cells expressed CD40 and there was no influence of LMP1 transfection on the expression of this marker (Table 2).

Finally, we observed that transfection of cells with both LMP1 variants and empty vector increased the number of cells expressing the immunosuppressive molecule PD-L1 (from 17% in untransfected control) to more than 37.9% in LMP1-transfected cells (figure 13C).

Cell Death analysis in NP69-LMP1 cells

In order to investigate whether cell transfection could change the cell ability, we analyzed the number of apoptotic (Anexin V positive cells) and dead cells (7AAD positive cells) after transfection with different LMP1 variants.

We performed cell death analysis in NP69 cells, expressing or not LMP1, in order to evaluate if LMP1 variants are responsible for cell death by late apoptosis or necrosis, and if it could be related with tumor progression induced by LMP1. Details of cell death are represented at the Table 3.

Table 3: Cell death and apoptosis of NP69 cells transfected with LMP1.

	Molecules analyzed (%)	
Cell lines	Anexina	7AAD
NP69 (control)	10,8	9,92
NP69-pBabe	9,25	39,2
NP69-B95.8	10,0	41,5
NP69-Alaskan	10,2	38,5
NP69-Med+	9,44	23,5
NP69-China 1	9,22	26,0
NP69-China 2	9,78	25,7

We observed that NP69, expressing LMP1 variants (NP69-B95.8 – 41.5%; NP69-Alaskan – 38.5%; NP69-Med+ - 23.5%; NP69-China 1 – 25%; NP69-China 2 – 25.7%) shows higher percentage of cells dying by necrosis when compared to NP69 cells (9.92%) (figure 18). Anexin expression, as a late apoptosis marker, was similar, and in low levels in NP69 (10.8%), NP69-pBabe (9.25%) e NP69-LMP1 cells (NP69-B95.8 – 10%; NP69-Alaskan – 10.2%; NP69-Med+ - 9.44%; NP69-China 1 – 9.22%; NP69-China 2 – 9.78%) (Table 3).

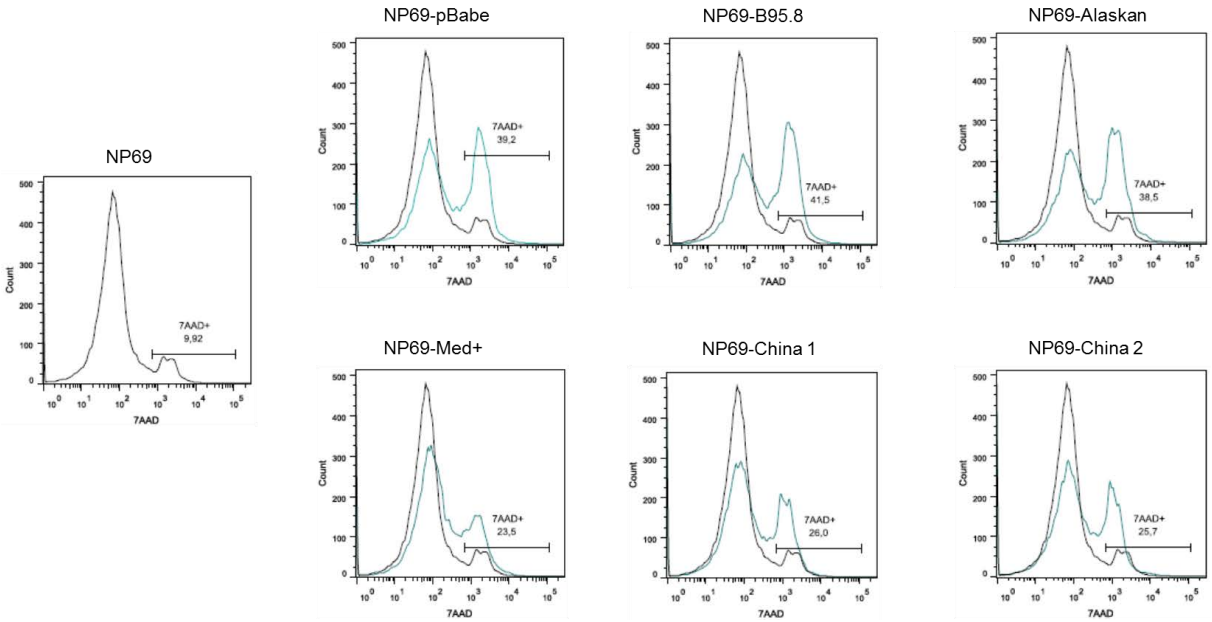

Figure 14: Flow cytometry analysis of cell death in NP69 cells. (A): Cytometry analysis of cell death by necrosis (7AAD). NP69, black line; NP69-pBabe e NP69-LMP1, blue line.

Discussion

This study focused on the role of LMP1 variants on two aspects of carcinogenesis, the migration and invasion properties of transformed cells, and the expression of selected surface markers.

First, transfection protocol should be validated by demonstration that cells transfected with each LMP1 variant was able to express this protein. It was demonstrated by western blot assay that all transfected cells were able to express hemaglutinin (HA), a protein which gene is concomitantly expressed with LMP1, as previously reported (Mainou BA, Raab-Traub N, 2006). In fact, only cells transfected with empty vector did not expressed HA, and it was observed for both HEK293T and NP69 cells. We also demonstrated that LMP1 was functional, since transfected cells were able to activate luciferase-labeled NF-κB vector, indicating that LMP1 was not under post-transfectional blockage, corroborating previous report showing high levels of NF- κB in cells transfected with some LMP1 variants such as Alaskan, Med+, China 1, and China 2[28].

Once the transfection protocol was validated, we analyzed whether LMP1 variants would be able to influence the migration ability of HEK293T and NP69. Our results show that HEK293T cells transfected with LMP1 variants did not shown different cellular migration pattern. These cells were used before just to analyze adhesion molecules involved in epithelial-mesenchymal transition, but have not been used to evaluate cell migration in scratch wound healing assay.

HEK293T cells were used in preliminary assays, but results with NP69 deserve more attention, since they mimic *in vitro* the EBV infection site. Our results on migration ability corroborate previous report that NP69 and other NPC cell lines have high migratory potential in the presence of LMP1[34,35,36]. As original data we found higher migration capacity in NP69-Med+, NP69-China 1 and NP69-China 2-transfected cells. Call us attention that these same variants also tended to show higher levels of NF-κB, an intracellular signaling factor involved in cell proliferation[37], and cell migration[38,39], enhancing the tumor progression. In fact, it was demonstrated that LMP1 stimulates invasive ability of NPC, leading to metastatic dissemination[40].

Scratch wound healing assay is based on a spontaneous cell growth and migration. However, the in vivo tumor development is usually stimulated by growth factors produced by tumor mesenchyme, endothelial cells or even immunocompetent or inflammatory cells (ref). Then, we decided to analyze the migration pattern in response to the

chemoattractant stimulus by endothelial growth factor (EGF), which is an important factor in induction of cellular proliferation of NPC cells[41]. We found that NP69 cells, expressing or not LMP1, responded equally to the EGF stimulus. It could be explained by the fact that NPC cells, and consequently NP69, commonly express EGFR. Thus, presence of EGF in the medium activated EGFR in NP69 cells, expressing or not LMP1, leading to a similar cellular migration pattern[42].

It leaded us to also evaluate invasion capacity in both cell lines. Our results with HEK293T cells show that only HEK293T-China 2 cells have higher invasion capacity than original HEK293T cells. Coincidently, it was reported that China 2 variant also induces a little bit higher levels of intracellular signaling pathways, such as NF-κB[34], which could explain why China 2 transfected cells has pronounced invasion capacity.

In spite of the result with HEK293T cells, NP69 cells invasion pattern analysis did not shown significant differences among the cells transfected with LMP1 variants, and the negative controls, NP69 and NP69-pBabe. Nonetheless, we can see a tendency to higher invasion capacity to NP69-China 2 cells, which is in accordance with results obtained with HEK293T cells.

The second aspect focused in this study was to understand whether and how LMP1 variants could work on the expression of surface molecules involved in immunomodulation. We analyzed expression of HLA-ABC and HLA-DR[43], ICAM – I (CD54)[44], CD40[45], CD80[46], CD83[47], and PD-L1, an immune suppressor factor upregulated in NPC EBV positive[48].

Normal epithelial cells express only HLA-ABC[43], whereas malignant cells usually decreased or lost HLA antigens to avoid immune system recognition[49]. Nevertheless, NPC-LMP1 positive cells express HLA-ABC and it could be upregulated by LMP1[50]. NP69 immunophenotyping shown that NP69 cells (negative control) had expression of HLA-ABC, and lack HLA-DR expression. NP69 cells after transfection with LMP1 variants did not alters this phenotype, cells kept the higher expression of HLA-ABC and the low expression of HLA-DR. Thus, our results indicated that LMP1 did not affect the expression of HLA antigens, but it not excluded the fact that these NP69-LMP1 cells avoid the immune system, since LMP1 variants could associate peptides with lower immunogenicity than others, avoiding an effective recognition by cytotoxic T cells[51].

Previous data show that LMP1 induce CD83 expression by NF-κB activation via CTAR1, and 2[48]. We found a tendency to upregulation of CD83 by LMP1 variants, with the greatest expression in NP69-Alaskan cells. These results are in accordance with previous study

that shown a strong immune response induced by LMP1 in B cells[48]. These strong immune response stimulated by LMP1 could be a mechanism used by EBV to maintain a pool of infected cells in a latent stage, and avoid viral clearance by the immune host.

It is also know that expression of CD80 molecule is induced by LMP1 through RelB translocation to the nucleus via CTAR1 and 2[47]. Our results found that only NP69-Alaskan cells shown a tendency to higher CD80 expression levels, all the other variants kept this molecule to the basal level observed in NP69 cells (an average of 1.5%). It is possible to suggest that LMP1 variant Alaskan is involved in CD83 and CD80 upregulation due to stimulation of NF-κB activation through their CTAR1 and/or 2.

It is known that CD54 (ICAM-I), a costimulatory molecule has low expression in normal epithelial cells, and is expressed in high levels in NPC cells[47]. It is also known that this expression could be induced by LMP1[51]. Unfortunately, different from previous reports, our results shown that NP69 cells express low levels of CD54, and LMP1 is not able to upregulate it independent of LMP1 variants.

It is important to note that LMP1 carboxy-terminal domain mimics CD40 intracellular activation. Both molecules stimulate downstream signaling pathways that lead to NF-κB activation, and B cell proliferation. However, the binding domain involved in this activation differs between the molecules[46.] CD40 is an important molecule involved in T lymphocyte priming and activation[47]. In opposition to the literature, our results show low levels of CD40 expression for both NP69 and NP69-LMP1 cells. Low levels of CD40 in NP69 cells could be explained by the fact that CD40 is expressed in early stages of differentiation in epithelial cells[52], thus this immortalized cell line should not express high levels of this molecule. For NP69-LMP1 cells, we can assume that these cells did not required CD40 upregulation in order to activate intracellular signaling pathway shared by both proteins because LMP1 mimics CD40 activity[46].

PD-L1 is an immune suppressor factor[49], and LMP1 is able to upregulated PD-L1 expression via JAK/STAT, AP-1, and NF-κB activation, leading to immune evasion[53]. We found that PD-L1 expression in NP69-LMP1 cells is high, and similar among all the variants analyzed. Interestingly, transfection with empty vector (pBabe) was sufficient to increase PD-L1 expression. Our results allows us to suggest that LMP1 expressed by NP69 cells upregulates PD-L1 expression. More experiments are required to elucidate whether LMP1 variants induced PD-L1 by different signaling pathways.

Finally, we analyzed NP69 cell death induced by LMP1. Previous reports showed that LMP1 transmembrane domain induced cell death by apoptosis in EBV-infected cells

lines. Notwithstanding, the carboxy-terminal moyet of LMP1 through UPR activation, and autophagy inhibit LMP1-induced apoptosis[54]. Our findings show that LMP1 transfected cells prevalently dead by necrosis. Even though this cell death process provoke an inflammatory process, we believe that it could promote extravasation of viral proteins, including the EBV oncoprotein LMP1 to tumor microenvironment to modulate *in situ* immune cells, endothelial cells, and fibroblasts, leading to an immune evasion, and proliferation of viable infected cells.

In summary, our results indicated that LMP1 is responsible for the changes in NP69-LMP1 positive cells morphology, for the stimulation of cell death by necrosis, and modulation of microenvironment cells, to facilitate immune evasion through modulation of CD80, CD83, and PD-L1. Furthermore, four LMP1 variants analyzed - Alaskan, Med+, China 1, and China 2 are capable of upregulate cellular migration at least through enhancing NF-κB activation. These data reinforce the view that LMP1 is a key oncoprotein involved in NPC carcinogenesis, especially in tumor progression, metastatic dissemination, and immune evasion. More experiments are required to assess which signaling pathways are involved in those LMP1 activity, and whether these pathways and/or molecules could be used as a therapeutic target.

Acknowledgements

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Conclusão

Nossos Resultados indicam que LMP1 é responsável por mudanças na morfologia de Células NP69-LMP1 positivas, pela estimulação de morte celular por necrose e modulação de células do microambiente tumoral, para facilitar a evasão imune. Esses dados reforçam a visão de que LMP1 está envolvida na carcinogênese do NPC, especialmente, na progressão tumoral, disseminação metastática e evasão imune.

Anexos

FACULDADE DE MEDICINA DE BOTUCATU -UNESP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Migração e invasão celular in vitro de células humanas expressando variantes da oncoproteína LMP1 do vírus de Epstein-Barr (EBV)

Pesquisador: Nathália Sutti Laszkiewicz

Área Temática:

Versão: 1

CAAE: 28906514.6.0000.5411

Instituição Proponente: Departamento de Patologia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 607.187

Data da Relatoria: 07/04/2014

Apresentação do Projeto:

O vírus Epstein-Barr (EBV) infecta latentemente mais de 90% da população humana. A infecção viral está associada ao desenvolvimento da mononucleose infecciosa e alguns cânceres, incluindo o linfoma de Burkitt endêmico africano, o carcinoma indiferenciado de nasofaringe, alguns casos de linfoma de Hodgkin clássico e desordens linfoproliferativas pós-transplante. A proteína latente de membrana 1 (Latent membrane protein 1 - LMP1) é o principal produto do EBV com propriedades transformantes, tanto in vitro quanto in vivo. Diferentes variantes de LMP1 são descritas, discriminadas principalmente por variações nos domínios transmembrana e carboxi-terminais da proteína. Até o momento não é sabido se diferentes variantes de LMP1 apresentam propriedades distintas no que se refere à progressão tumoral, sobretudo para tumores sólidos.

Objetivo geral

O presente estudo visa avaliar a hipótese de que diferentes variantes da LMP1 do EBV podem ter propriedades distintas no que se refere à indução de migração celular e potencial de invasão em células transfectadas in vitro, o que deve repercutir nos efeitos da infecção pelo EBV na progressão de cânceres associados aos vírus.

Objetivos específicos

1. Estabelecer modelo in vitro de células HEK293 expressando diferentes variantes de LMP1 do

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EBV;

2. Analisar e contrastar as propriedades in vitro (migração celular e capacidade de invasão) de HEK293 expressando diferentes variantes de LMP1 do EBV.

Métodos

As células HEK293 (células embrionárias de rim humano) serão transfectadas com vetores de expressão de diferentes variantes de LMP1 do EBV, gentilmente doados pelas Prof. Nancy Raab-Traub (University of North Carolina; Center for Infectious Diseases, Chapel Hill, NC, USA). A transfecção será realizada com reagente carreador não-lipossomal FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA). As células transfectadas serão cultivadas por aproximadamente 24-48h. Subsequentemente, serão submetidas à extração de proteínas para avaliação da expressão de LMP1 por imunoblots (Wes-tern Blotting – WB). Em seguida, serão selecionadas com puromicina (Sigma-Aldrich, St. Louis, MO, USA) para obtenção de linhagens estavelmente transfectadas para continuidade dos experimentos. Após serão realizadas as estimativa de motilidade celular e potencial de invasão in vitro. A análise estatística está bem descrita na página 15 do projeto.

Objetivo da Pesquisa:

Objetivo geral

O presente estudo visa avaliar a hipótese de que diferentes variantes da LMP1 do EBV podem ter propriedades distintas no que se refere à indução de migração celular e potencial de invasão em células transfectadas in vitro, o que deve repercutir nos efeitos da infecção pelo EBV na progressão de cânceres associados aos vírus.

Objetivos específicos

1. Estabelecer modelo in vitro de células HEK293 expressando diferentes variantes de LMP1 do EBV;

2. Analisar e contrastar as propriedades in vitro (migração celular e capacidade de invasão) de HEK293 expressando diferentes variantes de LMP1 do EBV.

Avaliação dos Riscos e Benefícios:

Não há riscos e os benefícios são: determinar padrões distintos de migração e invasão celular para as diferentes variantes de LMP1 do EBV, auxiliando na identificação de variantes mais propensas a uma característica mais agressiva, além de auxiliar em estudos futuros no que diz respeito à progressão tumoral.

FACULDADE DE MEDICINA DE BOTUCATU -UNESP

Continuação do Parecer: 607.107

Comentários e Considerações sobre a Pesquisa:

O presente trabalho é um estudo experimental (dissertação de mestrado) que irá utilizar células embrionárias de rim humano já disponíveis no laboratório do pesquisador. O estudo pretende determinar se diferentes variantes da LMP1 do EBV podem ter propriedades distintas na repercussão da infecção pelo EBV na progressão de cânceres associados aos vírus. Os autores solicitam dispensa do TCLE pois trata-se de um estudo com células embrionárias de rim humano (HEK293) disponíveis no laboratório do pesquisador, não sendo necessário recrutar voluntários doadores de células para o estudo. Concordo com a justificativa dos pesquisadores, no entanto, o pedido de dispensa foi solicitado apenas no documento de informações básicas do projeto. É necessário anexar documento separado com a solicitação. Os outros documentos necessários foram anexados ao processo. Tem orçamento financeiro de R$3230,00 para bolsa de estudo.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos obrigatórios foram anexados

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

O projeto pode ser aprovado sem ressalvas.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Projeto de Pesquisa APROVADO em reunião do CEP de 07/04/2014, sem necessidade de envio à CONEP.

Ao final do projeto é necessário enviar o Relatório Final de Atividades.

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Continuação do Parecer: 607.107

BOTUCATU, 07 de Abril de 2014

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