Aurica-Georgeta TELCIAN

TEZA DE DOCTORAT - REZUMAT

FIZIOLOGIA CELULELOR EPITELIALE RESPIRATORII IN RELATIE CU INFECTIA CU VIRUSUL RESPIRATOR SYNCITIAL

CUVINTE CHEIE

celule epiteliale bronhiale, complexul PD-Ls/PD-1, limfocite T $CD8^+$, virus respirator syncitial

INTRODUCERE

Complexul caii PD-Ls/PD-1 (Programmed Death Ligands –Programmed Death Receptor), consta din doi liganzi PD-L1 si PD-L2 si receptorul lor comun PD-1. PD-Ls situati pe celule de prezentare a antigenelor interactioneaza cu PD-1 de pe limfocitele T si participa la reglarea raspunsurilor din partea limfocitelor [1, 2]. In infectiile virale cronice, blocarea interactiunilor dintre PD-1 si PD-L1 a avut ca rezultat in experimente *ex vivo*, imbunatatirea functiilor limfocitelor T $CD8^+$ specifice fata de virus care erau extenuate [3-5] iar in experimente *in vivo*, a restaurant abilitatea limfocitelor T $CD8^+$ de a prolifera, de a secreta cytokine, de a omora celulele infectate si de a descreste incarcatura virala [6].

Rolul interactiunilor PD-Ls/PD-1 in infectiile virale acute este putin cunoscut. In cazul soriceilor KO pentru PD-1, cand au fost infectati cu adenovirus s-a constatat expansiunea limfocitelor T hepatice si un clearance viral mai rapid [7] iar in cazul infectiei acute cu virusul rabiei, absenta PD-1 a determinat un control mai bun al incarcaturii virale si o mai mare acumulare locala de limfocite T $CD8^+$ [8].

Virusul Respirator Syncitial (VRS) reprezinta cauza majora a bronsiolitelor acute [9], si a morbiditatii datorate bolior respiratorii la copii [10] si la persoanele in varsta [11]. Imunitatea pentru VRS este incompleta si reinfectii apar de-a lungul vietii. Persistenta VRS a fost raportata in soricei [12], porcusori de guineea [13] sobolani [14] si la oameni cu bronho-pneumonie cronica obstructiva [15]. Mecanismul pentru memoria imuna

deficienta in raspuns la infectia cu VRS ramane sa fie elucidata si necesita mai multe eforturi de cercetare.

Dupa trecerea printr-o primo-infectie respiratorie virala s-a estimat ca ~50% dintre limfocitele T $CD8^+$ cu memorie sunt limfocite numite "effector-memory" (EM) care persista in tesuturi non-limfoide [16-18]. Aceste limfocite T $CD8^+$ EM locale sunt implicate in raspunsul initial in cazul reinfectiilor, ajutate de mai multe limfocite T EM recrutate din circulatie [19].

Celulele epiteliale bronhiale (CEB) reprezinta tinta predilecta a infectiei cu VRS si exprima molecule asociate cu prezentarea antigenelor [20], inclusiv PD-Ls [21, 22]. Complexul caii PD-Ls/PD-1 se considera important in cadrul mecanismelor de aparare implementate de gazda si mule virusuri incearca sa exploateze aceasta cale inhibitorie prin PD-L1 pentru a descreste raspunsurile limfocitelor T si a favoriza persistenta patogenilor [23].

Ipoteza noastra a fost ca prin exprimarea moleculelor PD-Ls, CEB pot influenta direct limfocitele T $CD8^+$ EM locale. In consecinta, am investigat expresia moleculelor PD-Ls in celulele epiteliale respiratorii umane si influenta infectiei cu VRS asupra lor. De asemenea, intr-un sistem in care am co-cultivat CEB si limfocite T, am determinat influenta caii PD-Ls/PD-1 asupra functiilor antivirale a limfocitelor T $CD8^+$.

REZULTATE SI DISCUTII

PD-L1 este exprimat constitutiv pe CEB umane primare si expresia lui este amplificata de infectia cu VRS

Grupul nostru de cercetare a publicat in prealabil expresia moleculelor PD-L1 si PD-L2 in linii celulare epiteliale traheale, bronhiale si alveolare [22]. In lucrarea prezenta am extins aceste studii la celulele umane primare si am confirmat ca CEB umane primare exprima constitutiv PD-L1 dar nu si PD-L2.

Expunerea CEB umane primare la concentratii crescute de VRS a avut ca rezultat amplificarea expresiei molecule PD-L1 la nivel de ARN mesager si proteina de suprafata intr-un mod direct proportional cu doza de virus folosita. Amplificarea expresiei datorata infectiei cu VRS a fost dependenta de replicarea virala deoarece folosirea de VRS inactivat prin expunerea la lumina UV nu a avut nici un effect asupra expresiei moleculei

PD-L1. Expresia moleculei PD-L2 a fost amplificata de infectia cu VRS doar la nivelul ARN mesager, in timp ce proteina de suprafata a fost indetectabila, cel putin la 24 ore dupa infectia virala.

Infectia cu VRS a CEB activeaza si stimuleaza proliferarea limfocitelor T CD8$^+$

Dupa ce am demonstrat ca infectia cu VRS amplifica expresia PD-L1 in CEB umane primare, in continuare am investigat daca aceasta molecula este functionala si interactioneaza cu PD-1 de pe limfocitele umane T CD8$^+$. In acest scop am folosit un sistem de co-cultura *in vitro* in care celule BEAS-2B (linie celulara epiteliala bronhiala) infectate sau nu cu VRS, au interactionat direct cu limfocite umane T CD8$^+$.

Initial am confirmat rezultatele publicate anterior despre expresia PD-L1/PD-L2 in celulele BEAS-2B si amplificarea exprimarii lor de catre infectia cu VRS [22]. De asemenea am confirmat specificitatea anticorpilor blocanti pentru PD-L1/PD-L2. Cum expresia PD-L1/PD-L2 indusa de infectia cu VRS a celulelor BEAS-2B a fost prezenta intre 24-96 ore post infectia virala, CEB au fost infectate cu VRS si dupa 24 ore au fost co-cultivate cu limfocite T CD8$^+$ (momentul zero a fost considerat cand limfocitele au fost adaugate la CEB) si au fost urmarite pana la 72 ore dupa co-cultura, astfel incat inducerea expresiei moleculei PD-L1 de catre VRS a fost prezenta de-a lungul timpului cat CEB si limfocitele T au fost co-cultivate.

Prima data am determinat daca infectia cu VRS a CEB a dus la activarea limfocitelor T CD8$^+$ in co-cultura, prin determinarea expresiei CD69, CD25 si PD-1. Limfocitele T CD8$^+$ au exprimat markerii de activare CD69 si CD25 intr-un procent mai mare cand au fost co-cultivate cu CEB infectate cu VRS comparativ cu cele co-cultivate cu CEB neinfectate cu virus. Nu am gasit o diferenta semnificativa in procentul de limfocite T pozitive pentru PD-1. Activarea limfocitelor T nu a rezultat din stimularea lor directa de catre VRS, deoarece procentul limfocitelor T pozitive pentru CD69 cand au fost expuse singure la VRS a fost 2.4±0.8 %, similar cu limfocitele T co-cultivate cu CEB neinfectate cu VRS (2.1±0.3%) sau cu limfocitele T cultivate singure in mediul de cultura (1.9±0.8%, p=ns intre grupuri).

Cum limfocitele T CD8$^+$ prezente in epiteliul tractului respirator sunt mai probabil “effector-memory” (EM) sau “terminally-differentiated” (TD) [24], am investigat abilitatea CEB infectate cu VRS de a activa aceste doua subpopulatii specifice de

limfocite T $CD8^+$. Am gasit ca semnificativ mai multe celule T $CD8^+$ EM si TD exprima CD69 cand sunt co-cultivate cu CEB infectate cu VRS. Pentru CD25 diferenta nu a fost semnificativa insa procentul de limfocite T $CD8^+$ pozitive pentru PD-1 a fost mai mare pentru ambele subpopulatii in cazul expunerii limfocitelor T la CEB infectate cu VRS.

In continuare am comparat proliferarea limfocitelor T $CD8^+$ co-cultivate cu CEB neinfectate versus cele co-cultivate cu CEB infectate cu VRS prin marcarea lor cu CFSE (carboxyfluorescein diacetate succinimidyl ester). Procentul de limfocite T $CD8^+$ neproliferate dupa stimularea policlonala cu PHA (Phytohemagglutinin) a fost mai mare in cazul limfocitelor T $CD8^+$ co-cultivate cu CEB neinfectate cu VRS (94.8±1.2%) comparativ cu cele co-cultivate cu CEB infectate cu VRS (21.09±4.9%, n=4, $p<0.001$), unde celulele au proliferat formand intre 2-8 generatii noi. Rezultate similare am obtinut cand limfocitele T $CD8^+$ au fost pre-stimulate cu anti-CD3/IL-2 inainte de a fi adaugate in co-cultura.

Infectia cu VRS a CEB creste productia de cytokine antivirale si capacitatea cytotoxica a limfocitelor T $CD8^+$

Productia de IFN-γ si IL-2 sunt elemente cheie ale functiei anti-virale a limfocitelor T $CD8^+$. Frecventa limfocitelor T $CD8^+$ din co-cultura pozitive pentru IFN-γ si IL-2 au fost determinate prin flow-cytometrie (cytokinele au fost evidentiate intracelular in celulele restimulate in ultimele 4 ore inainte de determinare cu PMA si ionomycin in prezenta de brefeldin). Procentul limfocitelor T $CD8^+$ pozitive pentru IFN-γ sau IL-2 a fost crescut semnificativ cand limfocitele au fost co-cultivate cu CEB infectate cu VRS comparativ cu cele co-cultivate cu CEB neinfectate.

Analizand subpopulatiile efectoare ale limfocitelor T $CD8^+$, ambele EM si TD au avut procente semnificativ crescute de celule pozitive pentru IFN-γ, dar nu si pentru IL-2, cand au fost co-cultivate cu CEB infectate cu VRS comparativ cu cele co-cultivate cu CEB neinfectate.

Pentru a investiga mai amanuntit care dintre limfocitele T $CD8^+$ care au produs cytokinele, celulele pozitive au fost selectate si transpuse intr-un grafic pe baza markerilor care definesc subpopulatiile in cadrul limfocitelor T $CD8^+$. Am observat ca majoritatea limfocitelor care au produs IFN-γ se incadrau intre EM sau TD, cu foarte putine limfocite producatoare de IFN-γ apartinand subpopulatiilor naive sau “central

memory". In cazul limfocitelor producatoare de IL-2, majoritatea apartineau subpopulatiei naïve, un numar substantial erau EM si doar cateva se incadrau la subpopulatiile TD sau "central memory".

Am determinat de asemenea cantitatea de IFN-γ si IL-2 eliberata in supernatantii din co-culturi si am gasit o crestere semnifictiva a nivelului lor atunci cand limfocitele T $CD8^+$ au fost co-cultivate cu CEB infectate cu VRS comparativ cu cele co-cultivate cu CEB neinfectate. Nivelul maxim a fost atins la 48 ore dupa co-cultura, motiv pentru care am ales acest interval de timp sa facem majoritatea analizelor pentru experimentele noastre.

Cum activitatea cytotoxica este de asemenea o componenta importanta a functiei antivirale a limfocitelor T $CD8^+$, in continuare am investigat efectele CEB infectate cu VRS asupra activitatii cytotoxice a limfocitelor T. Procentul de limfocite T $CD8^+$ producatoare de granzyme B si perforin (determinate dupa utilizarea de brefeldin in ultimele 4 ore inainte de analiza) a inregistrat o crestere de amploare mai mica dar totusi semnificativa cand limfocitele au provenit din co-culturile cu CEB infectate cu VRS comparativ cu cele co-cultivate cu CEB neinfectate.

Cand au fost examinate subpopulatiile efectoare, in mod asemanator, cresteri mici dar semnificative au fost observate pentru procentele de limfocite T $CD8^+$ producatoare de granzyme B sau perforin, in cazul subpopulatiei TD cand limfocitele au fost co-cultivate cu CEB infectate cu VRS, fara diferente semnificative in cazul subpopulatiei EM. Analizand celulele pozitive pentru fiecare dintre aceste proteine cytotoxice, s-a observat ca majoritatea limfocitelor producatoare de granzyme B si perforin apartin subpopulatiilor TD si EM ale limfocitelor T $CD8^+$.

Granzyme B determinata ca proteina secreatata in supernatantii din co-culturile de limfocite T/CEB infectate cu VRS a fost de asemenea crescuta comparativ cu co-culturile de limfocite T/CEB neinfectate.

Blocarea PD-L1 determina cresterea nivelului de cytokine anti-virale si de proteine cytotoxice in cazul limfocitelor T $CD8^+$ co-cultivate cu CEB infectate cu VRS

Pentru a investiga rolul functional al caii PD-L1/PD-L2:PD-1 in cazul interactiunilor dintre CEB cu limfocitele T $CD8^+$, limfocitele T au fost co-cultivate cu CEB infectate cu VRS in prezenta de anticorpi blocanti pentru PD-L1. Nivelul de IFN-γ, IL-2 si granzyme B eliberate in supernatanti au fost crescute comparativ cu cele provenite din co-culturi

similare dar in absenta de anticorpi blocanti pentru PD-L1. In contrast, anticorpii blocanti pentru PD-L2 nu au avut nici o influenta asupra nivelului de IFN-γ, IL-2 si granzyme B secretate in supernatantii din co-culturile de limfocite T/CEB infectate cu VRS. Anticorpii de control nu au avut nici un efect asupra nivelului de cytokine secretate comparativ cu co-culturile de limfocite T/CEB infectate cu VRS fara anticorpi blocanti (IFN-γ 139.5±28.3 vs 129.6±22.5 pg/mL; IL-2 43.7±5.5 vs 35.1±4.5 pg/mL; granzyme B 15.7±3.8 vs 16.8±2.9 ng/mL, p=ns in toate cazurile).

Blocarea PD-L1 descreste expresia genica a VRS in CEB

In final am analizat replicarea VRS prin cuantificarea polimerazei virale (gena L) in CEB din sistemul de co-cultura si am observant o reducere semnificativa a expresiei genei L a VRS cand CEB infectate cu VRS au fost co-cultivate cu limfocite T $CD8^+$, comparativ cu CEB infectate cu VRS cultivate singure (40% reducere, $p<0.05$), confirmand ca limfocitele T $CD8^+$ au un effect antiviral in sistemul de co-cultura. Expresia genei L a VRS a fost scazuta si mai mult cand anticorpii blocanti pentru PD-L1 au fost adaugati in sistemul de co-cultura comparativ cu co-culturile fara anticorpi blocanti, in timp ce anticorpii de control nu au avut nici un effect. Cum era de asteptat, bazat pe faptul ca nu au indus productia de cytokine de tipul 1 sau productia de proteine cytotoxice, anticorpii blocanti pentru PD-L2 nu au influentat expresia genica a VRS in sistemul de co-cultura.

DISCUTII

Am raportat aici ca infectia cu VRS a CEB umane primare induce puternic expresia moleculei PD-L1 iar in cazul co-culturilor dintre limfocite T $CD8^+$ si CEB, cand CEB au fost infectate cu VRS au dus la activarea limfocitelor T, proliferarea lor si producerea de cytokine si proteine cytotoxice. Ultimele au fost observate in principal in subseturile EM si TD ale limfocitelor T $CD8^+$. Blocarea PD-L1 a dus la cresterea secretiei de IFN-γ, IL-2 si granzyme B si a suprimat replicarea VRS. Aceste rezultate sugereaza ca inducerea de catre virus a expresiei PD-L1 pe CEB inhiba functiile antivirale ale limfocitelor locale T $CD8^+$ in cazul unei infectii acute virale respiratorii.

PD-L1 este exprimat constitutiv in celule primare nazale si CEB [21, 22, 25]. Poly I:C si Rhinovirusul-16 induc ambele molecule PD-L1 si PD-L2 pe CEB primare si cele nazale [25]. Noi am gasit ca infectia cu VRS a CEB primare induce robust expresia PD-L1 de

~30-ori pentru ARN mesager si de ~4-ori pentru proteina. In contrast, Rinovirusul-16 induce proteina pentru PD-L1 de ~2-ori pe CEB umane primare [25]. Aceste date, impreuna cu efectul inhibitor cunoscut al PD-L1 in contextul infectiilor virale cronice [3-5], ne-a determinat sa studiem *in vitro* importanta functionala a inductiei moleculei PD-L1 pentru raspunsurile immune anti-virale in cazul ifectiei cu VRS.

Prima ipoteza a fost ca celulele epiteliale neinfectate nu o sa activeze limfocitele T $CD8^+$, dar in contrast, CEB infectate cu VRS o sa stimuleze limfocitele T $CD8^+$ sa exprime markeri ai activarii, sa prolifereze si sa secrete cytokine si proteine cytotoxice.

CD69 este unul dintre cei mai timpurii markeri ai activarii exprimati de limfocitele T. In raspuns la stimularea allogenica, maximul de expresie al CD69 a fost raportat a fi <3% [26]. In concordanta cu aceasta, in studiile noastre CD69 a fost prezent pe 2-3% dintre limfocitele T co-cultivate cu CEB neinfectate, dar acest procent a fost crescut semnificativ de ~3 ori pe limfocitele T co-cultivate cu CEB infectate cu VRS. O inductie mai slaba a fost inregistrata pentru CD25 si PD-1 (care este crescut in mod persistent pe limfocitele extenuate in cazul infectiilor virale cornice) [3-5]. PD-1 a fost gasit in procente scazute (<0.5%) pe limfocitele T $CD8^+$ folosite in experimetele noastre intrucat acestea au fost izolate de la donatori normali si nu a fost semnificativ crescut pe limfocitele T considerate in ansamblu cand au fost co-cultivate cu CEB infectate cu VRS.

Limfocitele T $CD8^+$ situate mai probabil in contact cu CEB *in vivo* sunt subpopulatiile EM si TD [16, 18]. Am observat ca nivelurile de expresie pentru fiecare dintre CD69, CD25 sau PD-1 au fost mai crescute in aceste subpopulatii decat in limfocitele T luate in ansamblu cand acestea au fost co-cultivate cu CEB neinfectate, iar inductia lor a fost mai mare cand au fost co-cultivate cu CEB infectate cu VRS. Aceste date sugereaza ca limfocitele T $CD8^+$ care raspund la CEB infectate cu VRS prin activare sunt in principal subseturile EM si TD.

Limfocitele T $CD8^+$ stimulate cu PHA nu au proliferat in co-cultura cu CEB neinfectate. In cazul celor co-cultivate cu CEB infectate cu VRS, majoritatea au proliferat cel putin o generatie. Limfocitele T $CD8^+$ stimulate policlonal si cultivate singure au proliferat intr-o maniera similara cu cele co-cultivate cu CEB infectate cu VRS, sugerand ca epiteliul neinfectat are capacitatea de a inhiba proliferarea limfocitelor T $CD8^+$, aceste rezultate fiind similare cu date publicate din experimente *in vitro* folosind celule de la soricei [27].

Productia de cytokine (IFN-γ/IL-2) si de proteine cytotoxice (granzyme/perforin) a inregistrat un tipar similar cu cel de la markerii de activare, cu o productie mai mare in cazul limfocitelor T $CD8^+$ co-cultivate cu CEB infectate cu VRS comparativ cu cele co-cultivate cu CEB neinfectate. Productia de IFN-γ in limfocitele T $CD8^+$ a avut loc in cea mai mare masura in limfocitele apartinand subpopulatiilor efectoare, in timp ce productia de IL-2 a avut loc in principal in celulele din subpopulatia naiva. Productia de granzyme B si perforin a fost gasita aproape exclusiv in subpopulatiile TD si EM. Limfocitele T au exprimat proteine cytotoxice intr-un procent mare chiar si cand au fost co-cultivate cu CEB neinfectate si in consecinta cresterea procentuala a celulelor pozitive atunci cand au fost co-cultivate cu CEB infectate cu VRS a fost relativ modesta.

Aceste rezultate au fost in concordanta cu functiile anticipate, cu CEB in conditii normale ne-activand limfocitele T, dar in conditiile infectiei cu VRS, CEB vor stimula limfocitele T $CD8^+$ locale, echipandu-le pentru expansiune si functii efectoare anti-virale.

In continuare am ipotezat ca expresia moleculei PD-L1 crescand in contexul infectiei virale o sa previna activarea completa a functiilor antivirale ale limfocitelor. Intr-adevar, am gasit o crestere suplimentara a productiei de IFN-γ, IL-2 si granzyme B in prezenta de anticorpi blocanti pentru PD-L1 (dar nu si pentru PD-L2), confirmand ca expresia crescuta a PD-L1 joaca un rol inhibitor in acest sistem.

Exista putine modele care investigheaza “in direct” functiile limfocitelor T cu memorie in raspunsul fata de infectiile virale. Astfel am conceput acest model de co-cultura *in vitro* care sa ne permita studierea acestor evenimente intr-un sistem asemanator cu interactiunile dintre celulele epiteliale si limfocitele T asa cum se intampla *in vivo*. Limfocitele T se gasesc in asociatie cu epiteliul din tractul respirator si a fost demonstrat ca celulele epiteliale bronhiale umane au capacitatea de a prezenta direct antigene si astfel activeaza direct limfocitele cytotoxice T $CD8^+$ [28]. Exista evidente suficiente care arata ca limfocitele T $CD8^+$ effector-memory nu prolifereaza in plamani sau caile respiratorii si sunt recrutate in continuu din circulatie [19]. Astfel, prin folosirea de limfocite T $CD8^+$ izolate din sange periferic noi am incercat sa recreem un mediu inconjurator similar cu cel gasit in tractul respirator *in vivo*. Rezultatele noastre confirma faptul ca PD-L1 poate juca un rol inhibitor in cazul interactiunilor anti-virale locale intre limfocitele T si CEB in cazul infectiilor virale acute respiratorii.

Aceste date pot avea implicatii importante in susceptibilitatea crescuta a copiilor la infectia cu VRS, si de asemenea in cazul memoriei imune incomplete la infectia cu VRS de la copiii mai mari, adulti sau varstnici. Blocarea expresiei PD-L1 poate deveni o alternativa terapeutica in incercarea de a stimula imunitatea anti-virala in cadrul infectiilor virale respiratorii acute cum este si cea cu VRS.

1. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol*. 2005; 23:515-48.
2. Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol*. 2007; 19(3):309-14.
3. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006; 443(7109):350-4.
4. Penna A, Pilli M, Zerbini A, Orlandini A, Mezzadri S, Sacchelli L, Missale G, Ferrari C. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology*. 2007; 45(3):588-601.
5. Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertoletti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol*. 2007; 81(8):4215-25.
6. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006; 439(7077):682-7.
7. Iwai Y, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med*. 2003; 198(1):39-50.
8. Lafon M, Megret F, Meuth SG, Simon O, Velandia Romero ML, Lafage M, Chen L, Alexopoulou L, Flavell RA, Prehaud C, Wiendl H. Detrimental contribution of the immuno-inhibitor B7-H1 to rabies virus encephalitis. *J Immunol*. 2008; 180(11):7506-15.
9. Hull J, Thomson A, Kwiatkowski D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. *Thorax*. 2000; 55(12):1023-7.
10. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P. The burden of respiratory syncytial virus infection in young children. *N Engl J Med*. 2009; 360(6):588-98.
11. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med*. 2005; 352(17):1749-59.
12. Schwarze J, O'Donnell DR, Rohwedder A, Openshaw PJ. Latency and persistence of respiratory syncytial virus despite T cell immunity. *Am J Respir Crit Care Med*. 2004; 169(7):801-5.
13. Hegele RG, Hayashi S, Bramley AM, Hogg JC. Persistence of respiratory syncytial virus genome and protein after acute bronchiolitis in guinea pigs. *Chest*. 1994; 105(6):1848-54.
14. Sorkness RL, Gern JE, Grindle KA, Mosser AG, Rosenthal LA, Mikus LD, Lemanske RF, Jr. Persistence of viral RNA in 2 rat strains differing in susceptibility to postbronchiolitis airway dysfunction. *J Allergy Clin Immunol*. 2002; 110(4):607-9.
15. Wilkinson TM, Donaldson GC, Johnston SL, Openshaw PJ, Wedzicha JA. Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2006; 173(8):871-6.
16. Ely KH, Cauley LS, Roberts AD, Brennan JW, Cookenham T, Woodland DL. Nonspecific recruitment of memory CD8+ T cells to the lung airways during respiratory virus infections. *J Immunol*. 2003; 170(3):1423-9.
17. Ostler T, Hussell T, Surh CD, Openshaw P, Ehl S. Long-term persistence and reactivation of T cell memory in the lung of mice infected with respiratory syncytial virus. *Eur J Immunol*. 2001; 31(9):2574-82.
18. Kohlmeier JE, Woodland DL. Immunity to respiratory viruses. *Annu Rev Immunol*. 2009; 27:61-82.
19. Hikono H, Kohlmeier JE, Ely KH, Scott I, Roberts AD, Blackman MA, Woodland DL. T-cell memory and recall responses to respiratory virus infections. *Immunol Rev*. 2006; 211:119-32.
20. Papi A, Stanciu LA, Papadopoulos NG, Teran LM, Holgate ST, Johnston SL. Rhinovirus infection induces major histocompatibility complex class I and costimulatory molecule upregulation on respiratory epithelial cells. *J Infect Dis*. 2000; 181(5):1780-4.

21. Kim J, Myers AC, Chen L, Pardoll DM, Truong-Tran QA, Lane AP, McDyer JF, Fortuno L, Schleimer RP. Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. *Am J Respir Cell Mol Biol*. 2005; 33(3):280-9.
22. Stanciu LA, Bellettato CM, Laza-Stanca V, Coyle AJ, Papi A, Johnston SL. Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines. *J Infect Dis*. 2006; 193(3):404-12.
23. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol*. 2007; 8(3):239-45.
24. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999; 401(6754):708-12.
25. Heinecke L, Proud D, Sanders S, Schleimer RP, Kim J. Induction of B7-H1 and B7-DC expression on airway epithelial cells by the Toll-like receptor 3 agonist double-stranded RNA and human rhinovirus infection: In vivo and in vitro studies. *J Allergy Clin Immunol*. 2008; 121(5):1155-60.
26. Craston R, Koh M, Mc Dermott A, Ray N, Prentice HG, Lowdell MW. Temporal dynamics of CD69 expression on lymphoid cells. *J Immunol Methods*. 1997; 209(1):37-45.
27. Wang H, Su Z, Schwarze J. Healthy but not RSV-infected lung epithelial cells profoundly inhibit T cell activation. *Thorax*. 2009; 64(4):283-90.
28. Kraetzel K, Stoelcker B, Eissner G, Multhoff G, Pfeifer M, Holler E, Schulz C. NKG2D-dependent effector function of bronchial epithelium-activated alloreactive T-cells. *Eur Respir J*. 2008; 32(3):563-70.

CURRICULUM VITAE

(Aprilie 2010)

DETALII PERSONALE:

Nume: Aurica Georgeta TELCIAN

Data nasterii: 9 Septembrie 1974

Nationalitatea: romana

E-mail: a.telcian@imperial.ac.uk

EDUCATIE:

- 1993-1999: Facultatea de Medicina Generala, Universitatea de Medicina si Farmacie "Iuliu Hatieganu" Cluj-Napoca, Romania – Diploma de Doctor-Medic emisa in 1 Octombrie 1999
- 2000-2006: Rezidentiatul in Medicina de Laborator, Universitatea de Medicina si Farmacie "Iuliu Hatieganu" Cluj-Napoca, Romania – Certificatul de Medic Specialist in Medicina de Laborator emis in 25 Mai 2006
- 2005-2010: Studenta la Doctorat (forma fara frecventa), Universitatea de Medicina si Farmacie "Iuliu Hatieganu" Cluj-Napoca, Romania

EXPERIENTA DE LUCRU:

- Ianuarie 2000-Februarie 2001: Medic Stagiar, Spitalul Municipal Cluj-Napoca, Romania
- Martie 2001-Octombrie 2002: Rezident Medicina de Laborator, Microbiologie, Spitalul de Infectioase Cluj-Napoca, Romania
- Noiembrie 2002-Iunie 2003: Rezident Medicina de Laborator, Biochimie, Clinica Medicala II, Cluj-Napoca, Romania
- Iulie 2003-Iulie 2005: Rezident Medicina de Laborator, Hematologie, Institutul Oncologic "Ion Chiricuta", Cluj-Napoca, Romania
- Iulie 2005-August 2008: Academic visitor, Departamentul de Medicina Respiratorie, Imperial College London, UK

- August 2008-prezent: Research Assistant, Departamentul de Medicina Respiratorie, Imperial College London, UK

PUBLICATII:

- Aurica G Telcian, Adriana Muresan, Vasile-Laza Stanca, Sebastian L Johnston, Luminita A Stanciu, "$CD8^+$ T cell cytokine & cytotoxic protein production and proliferation is differentially regulated by normal and RSV-infected human bronchial epithelial cells", Clujul Medical, vol 83, no 1:64-68, 2010
- Aurica G Telcian, Adriana Muresan, Vasile-Laza Stanca, Sebastian L Johnston, Luminita A Stanciu, "Gene expression of Programmed Death Ligand (PD-L)-1, PD-L2, Inducible Costimulator Ligand and B7-H3 on human alveolar epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines", Fiziologia, vol 19.3 (63):4-7, 2009
- Aurica G Telcian, Vasile-Laza Stanca, Michael R Edwards, James A Harker, Hongwei Wang, Nathan W Bartlett, Patrick Mallia, Mihnea T Zdrenghea, Tatiana Kebadze, Anthony J Coyle, Peter JM Openshaw, Luminita A Stanciu, Sebastian L Johnston, "RSV-induced bronchial epithelial cell PD-L1 expression inhibits $CD8^+$T cell anti-viral activity" [*manuscript in curs de preparare*]
- Zdrenghea MT, Laza-Stanca V, Telcian A, Johnston SL, Stanciu LA. "Respiratory epithelial cell calcitriol metabolism modulation by viruses: putative role in virus-induced asthma exacerbations", J Rom Soc Allergol Clin Immunol 2007;4.
- Popa L, Telcian A.G. "Molecular bases of new strategies in the treatment of cancer", Rev Obstetric Gynecol 1996; 44: 23-26.

PREZENTARI ORALE LA CONGRESE:

- "Calcitriol increases interferon stimulated gene expression and has anti-viral and anti-inflammatory activity in human bronchial epithelial cells", The XXVIII Congress of the EAACI, Varsovia, Polonia, 6-10 Iunie 2009

- "Respiratory Syncytial Virus negatively modulates vitamin D action in respiratory epithelial cells", The XXVII Congress of the EAACI, Barcelona, Spania, 7-11 Iunie 2008
- "Presentation of a fellowship experience in the United Kingdom", ERS Congress, Stockholm, Suedia, 15-19 Septembrie 2007
- "Functional effect of RSV-induced PD-L1 on epithelial cell activation of $CD8^+$T cells", ATS Congress, San Francisco, Statele Unite ale Americii, 18-23 Mai 2007, premiat cu un International Trainee Travel Award
- "RSV modulation of co-stimulatory molecules on primary human bronchial epithelial cells", Scoala de Vara EAACI-GA2LEN, Chalkidiki, Grecia, 23-27 Iunie 2006
- "Primary extramedullary, pleuropericardial, myeloid tumour. Case report and literature review"; Sesiunea Jubiliara a Institutului de Cancer "Ion Chiricuta" Cluj-Napoca, 6-9 Octombrie 2004, Jurnalul Societatii, pagina 43

PREZENTARI DE POSTERE:

- A.G. Telcian, L.A. Stanciu and S.L. Johnston. "Effector memory $CD8^+$T cell responses to RSV infected respiratory epithelial cells", ERS Congress, Viena, Austria, 12-16 Septembrie 2009
- Aurica G Telcian, Luminita A Stanciu and Sebastian L Johnston. "Calcitriol increases interferon stimulated gene expression and has antiviral and anti-inflammatory activity in human bronchial epithelial cells", ATS Congress, San Diego, Statele Unite ale Americii, Mai 2009
- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S.L. Johnston. "The role of RSV infected epithelial cells on $CD8^+$T cell responses", EAACI-GA2LEN Allergy School, Cluj-Napoca, Romania, 5-10 Septembrie 2007
- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S.L. Johnston. "$CD8^+$T cell responses to RSV infected respiratory epithelial cells", The XXVI Congress of the EAACI, Goteborg, Suedia, 9-13 Iunie 2007 – premiat cu un Junior Poster Prize
- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S. L. Johnston. "Functional effect of RSV-induced PD-L1 on epithelial cell activation of $CD8^+$T cells", The 5^{th} EAACI-

GA2LEN-DAVOS meeting, Basic Immunology on Allergy and Clinical Immunology, Davos, Elvetia, 1-4 Februarie 2007

- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S. L. Johnston. "Expression of PD-L1 on primary human bronchial epithelial cells and its regulation by Respiratory Syncytial Virus, IFN-γ and IL-4", The XXV Congress of the EAACI, Viena, Austria, 10-14 Iunie 2006 – premiat cu un Junior Poster Prize

ABSTRACTE IN COLABORARE:

- Sarah Essilfie-Quaye, Andy Durham, Aurica Telcian, Joseph Footitt, Ian Adcock and Sebastian Johnston. "Rhinovirus-induced HDAC2 nitration and reduced HDAC2 activity in THP-1 cells", ERS Congress, Septembrie 2010 [*submis*]
- J.Footitt, MB Trujillo-Toralbo, A. Telcian, P. Mallia & SL Johnston. "Changes in FEV1 and lower respiratory tract scores in a human model of rhinovirus induced COPD exacerbations", 2nd International Congress on Exacrebation of Airway Disease, Miami, Aprilie 2010 [*submis*]
- GN Feketea, AG Telcian, MR Edwards, LA Stanciu and SL Johnston. "IL-4 modulation of rhinovirus-induced CCL20/MIP-3a production in bronchial epithelial cells", ERS Congress, Viena, Austria, 12-16 Septembrie 2009
- LA Stanciu, N Rushwan, A Telcian, M Zdrenghea, SL Johnston. "Rhinovirus infection differentially modulates MHC class I-related chain (MIC) A and B molecules in human respiratory epithelial cells", ERS Congress, Viena, Austria, 12-16 Septembrie 2009
- G Feketea, AG Telcian, LA Stanciu, SL Johnston. "CCL20 production in bronchial epithelial cells is increased by rhinovirus infection" The XXVIII Congress of the EAACI, Varsovia, Polonia, 6-10 Iunie 2009 [prezentare orala]
- LA Stanciu, AG Telcian, MT Zdrenghea, V Laza-Stanca, SL Johnston. "The effect of calcitriol on VDR, RANTES and virus gene expression in rhinovirus-infected respiratory bronchial epithelial cells" The XXVII Congress of the EAACI, Barcelona, Spania, 7-11 Iunie 2008
- Luminita A. Stanciu, Cinzia M. Bellettato, Mihnea T. Zdrenghea, Vasile Laza-Stanca, Aurica G.Telcian, et al. "RSV modulation of co-stimulatory molecules on

human respiratory epithelial cells", American Association of Immunologists Meeting, Boston, Mai 2006

BURSE:

- Scholarship Award de la UCB Institute of Allergy (session of April 2005) pentru 6 luni la Imperial College London, Departamentul de Medicina Respiratorie
- Long Term Research ERS Fellowship (Mai 2006) pentru 1 an la Imperial College London, Departamentul de Medicina Respiratorie
- HC Roscoe (2009) Grant – British Medical Association, Dr Aurica Telcian - Imperial College London, "Virus infection of epithelial cells modulates molecules regulating cytotoxity: role in asthma exacerbations"

LIMBI STRAINE: Engleza/Franceza: scris/vorbit

Aurica-Georgeta TELCIAN

PhD THESIS - SUMMARY

PHYSIOLOGY OF RESPIRATORY EPITHELIAL CELLS RELATED TO RESPIRATORY SYNCYTIAL VIRUS INFECTION

KEY WORDS

bronchial epithelial cells, $CD8^+$ T cells, PD-Ls/PD-1 pathway, Respiratory Syncitial Virus

INTRODUCTION

The programmed death ligand (PD-L)-PD-1 receptor pathway, consists of the two ligands PD-L1 and PD-L2 and their common receptor PD-1. PD-Ls on antigen presenting cells interact with PD-1 on T cells and regulate T cell responses [1, 2]. In chronic viral infections, blocking PD-1 engagement to PD-L1 revived the *ex vivo* function of exhausted virus-specific $CD8^+$ T cells [3-5] and *in vivo*, restored $CD8^+$ T cell ability to proliferate, secrete cytokines, kill infected cells and decrease viral load [6].

The role of PD-Ls/PD-1 interactions in acute viral infection is poorly understood. When PD-1 KO mice were infected with adenovirus, there was hepatic T cell expansion and more rapid viral clearance [7] and during acute rabies virus infection, absence of PD-L1 led to better control of virus and a greater local accumulation of $CD8^+$ T cells [8]. The role of PD-1:PD-L1 pathway in acute respiratory viral infections is unknown.

Respiratory syncytial virus (RSV) is the major cause of acute bronchiolitis in infants [9], and respiratory morbidity in children [10] and the elderly [11]. Immunity to RSV is incomplete and re-infections occur throughout life. RSV persistence has also been reported in mice [12], guinea pigs [13] and rats [14] and humans with chronic obstructive pulmonary disease [15]. The mechanisms for the deficient memory immune response to RSV infection remain un-elucidated and demand further research effort.

Following recovery from a primary respiratory viral infection, it is estimated that ~50% of memory $CD8^+$ T cells are effector-memory T cells that persist in non lymphoid tissues [16-18]. These local effector-memory $CD8^+$ T cells are involved in the initial response to re-infection, assisted by more effector-memory $CD8^+$ T cells recruited from the circulation [19].

Bronchial epithelial cells (BECs) are the primary site of RSV infection and express molecules associated with antigen presentation [20], including the PD-Ls [21, 22]. The PD-Ls/PD-1 pathway is believed to be important in host defence as many viruses exploit the inhibitory PD-L1 pathway to down-regulate T cell responses and facilitate pathogen persistence [23].

We reasoned that by expressing PD-L molecules, BECs may directly influence local effector-memory $CD8^+$ T cells. We investigated PD-Ls expression in human airway epithelial cells and their induction by RSV. Also, in a BEC/T cell co-culture system we determined the influence of PD-1-PD-L pathways on $CD8^+$ T cell anti-viral functions.

RESULTS AND DISCUSSION

PD-L1 is constitutively expressed by human primary BECs and is up-regulated by RSV infection

Our group previously reported PD-L1 and PD-L2 expression in tracheal, bronchial and alveolar epithelial cell lines [22]. Here we extended these studies to human primary cells and confirmed that human primary BECs constitutively expressed PD-L1 but not PD-L2. Exposure of human primary BECs to increasing doses of RSV up-regulated PD-L1 mRNA expression and surface protein levels in a dose-responsive manner. RSV up-regulation of PD-L1 was replication-dependent (UV-inactivated RSV had no effect on PD-L1 expression). PD-L2 mRNA expression was up-regulated by RSV but surface protein was not found at 24h post-infection**.**

RSV infection of BECs activates and increases proliferation of CD8$^+$ T cells

Having demonstrated that RSV up-regulates PD-L1 expression on human primary BECs, we next wanted to investigat whether this molecule was functional in interacting with human CD8$^+$ T cells. We used an *in vitro* co-culture system in which RSV-infected BEAS-2B cells directly interacted with CD8$^+$ T cells.

We initially confirmed our previous findings [22] that BEAS-2B cells express PD-L1/PD-L2 and that RSV up-regulated them. We also confirmed the specificities of blocking antibodies to PD-L1 and PD-L2. As RSV-induced PD-L1/PD-L2 expression in these cells was present from 24hrs till 96hrs, BECs were infected with RSV alone for 24hrs and then co-cultured for 72hrs, so that induced PD-L expression would occur during BEC/CD8$^+$ T cell co-cultures.

We first determined if RSV infection of BECs resulted in CD8$^+$ T cell activation in co-culture by determining CD69, CD25 and PD-1 levels. CD8$^+$ T cells expressed the activation markers CD69 and CD25 significantly more when co-cultured with RSV-infected BECs compared to co-culture with un-infected BECs. There was no significant difference in PD-1-positive-CD8$^+$ T cells. CD8$^+$ T cell-activation was not a result of direct stimulation by RSV, as CD69$^+$CD8$^+$ T cells exposed to RSV alone were 2.4±0.8 %, compared to CD8$^+$ T cell co-cultured with un-infected BECs (2.1±0.3%) or CD8$^+$ T cells in media alone (1.9±0.8%, p=ns between groups).

As CD8$^+$ T cells present in the respiratory epithelium are likely to be either effector-memory (EM) or terminally-differentiated (TD) cells [24], we investigated the ability of RSV-infected BECs to activate these two specific subpopulations. Significantly more EM and TD CD8$^+$ T cells expressed CD69 when co-cultured with RSV-infected BECs

compared to EM or TD $CD8^+$ T cells in co-culture with un-infected BECs. There was no significant difference in CD25-positive but frequencies of PD-1-positive cells were higher in both $CD8^+$ T cell subpopulations during exposure to RSV-infected BECs.

We next compared proliferation of CFSE(luorescein diacetate succinimidyl ester)-labelled $CD8^+$ T cells in co-culture with un-infected versus RSV-infected BECs. Percentage of non-proliferating polyclonally (PHA-Phytohemagglutinin)-stimulated cells was higher in $CD8^+$ T cells co-cultured with un-infected BECs (94.8±1.2%) compared to co-cultures with RSV-infected BECs (21.09±4.9%, n=4, $p<0.001$), where cells underwent proliferation of 2-8 generations. Similar results were obtained when $CD8^+$ T cells were pre-stimulated with anti-CD3/IL-2 before being added to the co-culture.

RSV infection of BECs increases anti-viral cytokine production and cytotoxic capacity in $CD8^+$ T cells

IFN-γ and IL-2 production are key elements of $CD8^+$T cell anti-viral function. The frequencies of IFN-γ^+CD8^+ and IL-2^+CD8^+ T cells in co-culture were determined by intracellular staining (PMA and ionomicyn in the presence of brefeldin were used for the last 4hrs before staining). Percentage of $CD8^+$ T cells positive for IFN-γ or IL-2 was significantly higher when cells were co-cultured with RSV-infected BECs compared to un-infected BECs.

Among the effector subpopulations, both EM and TD $CD8^+$ T cells had significantly increased frequencies of IFN-γ^+, but not of IL-2^+ cells, when co-cultured with RSV-infected BECs compared to un-infected BECs.

To further investigate $CD8^+$ T cell subpopulations producing each cytokine, we back-gated $CD8^+$ T cells producing each cytokine and plotted these positive cells with markers for $CD8^+$ T cell subpopulations. When co-cultured with RSV-infected BECs, the majority of IFN^+CD8^+ T cells were EM and TD, while very few were naïve or central memory. The majority of IL-2-producing cells were naïve, though substantial numbers were EM with smaller numbers being TD or central memory.

We also assessed IFN-γ and IL-2 release into supernatants from the co-cultures and found a significant increase in IFN-γ- and IL-2-levels when $CD8^+$ T cells were co-cultured with RSV-infected BECs compared to un-infected BECs. The peak levels were at 48hrs post co-culture, therefore we used this time point to determine most of the outcomes.

As cytotoxic activity is also an important anti-viral function of $CD8^+$ T cells, we next investigated how RSV-infected BECs influence $CD8^+$ T cell cytotoxic activity. Frequencies of granzyme B- and perforin-positive $CD8^+$ T cells (only bfrefeldin was used for the last 4hrs before staining) were marginally, but significantly greater in co-culture with RSV-infected BECs compared to un-infected BECs.

When effector subpopulations were examined, similar small but significant increases in granzyme B- and perforin-positive cells were observed among TD $CD8^+$ T cells when co-cultured with RSV-infected BECs, however no significant differences were observed in the EM populations. Back-gating the $CD8^+$ T cells producing each cytotoxic protein from un-infected and RSV-infected BEC co-cultures, we confirmed that the majority of granzyme B- and perforin-producing cells were EM and TD $CD8^+$ T cells.

Granzyme B protein release into supernatants of co-cultured $CD8^+$ T cell/RSV-infected BECs was also increased compared to $CD8^+$ T cell/un-infected BEC co-cultures.

PD-L1 blockade increases anti-viral cytokine and cytotoxic protein levels in $CD8^+$ T cells co-cultured with RSV-infected BECs

To investigate the functional roles of PD-L1/PD-L2:PD-1 pathways in BEC/$CD8^+$ T cell interactions, $CD8^+$ T cells were co-cultured with RSV-infected BECs in the presence of blocking antibodies to PD-L1. These co-cultures released increased IFN-γ-, IL-2- and granzyme B-levels into supernatants, compared to co-cultures in the absence of anti-PD-L1 antibodies. In contrast, anti-PD-L2 antibodies had no influence on IFN-γ, IL-2 or granzyme B released in $CD8^+$ T cell/RSV-infected BEC co-cultures. The isotype controls showed no effect on cytokines released compared to $CD8^+$ T cells co-cultured with RSV-infected BECs with no blocking antibodies (IFN-γ 139.5±28.3 vs 129.6±22.5 pg/mL; IL-2 43.7±5.5 vs 35.1±4.5 pg/mL; granzyme B 15.7±3.8 vs 16.8±2.9 ng/mL, p=ns in all cases).

PD-L1 blockade decreases RSV gene expression in BECs

Finally, we assessed RSV-replication by quantifying RSV polymerase L gene expression in BECs in the co-culture system and observed a significant reduction in RSV L gene expression when RSV-infected BECs were co-cultured with $CD8^+$ T cells, compared to RSV-infected BECs alone (40% reduction, $p<0.05$), confirming that $CD8^+$ T cells have an anti-viral effect in this co-culture system. RSV L gene expression was further decreased

in the presence of PD-L1 blocking antibodies in co-cultures compared with RSV-infected BECs/$CD8^+$T cells without blocking antibody while the isotype control antibody had no effect. As expected based on their failure to influence type 1 cytokine and cytotoxic protein production, PD-L2 blocking antibodies did not influence the RSV gene expression in co-culture.

DISCUSSION

We report here that RSV infection of primary human BECs strongly up-regulated PD-L1 expression and that in $CD8^+$ T cell/BEC co-cultures, RSV-infected BECs increased $CD8^+$ T cell activation, proliferation and anti-viral cytokine and cytotoxic protein production. The latter were observed chiefly among effector-memory and terminally-differentiated $CD8^+$ T cell subsets. PD-L1 blockade enhanced $CD8^+$ T cell secretion of IFN-γ, IL-2 and granzyme B and suppressed RSV replication. These data indicate that virus induction of PD-L1 expression on BECs inhibits local $CD8^+$ T cell anti-viral activities in the context of an acute respiratory viral infection.

PD-L1 is constitutively expressed in primary nasal and BECs [21, 22, 25]. Poly I:C and rhinovirus-16 induce both PD-L1 and PD-L2 on primary BECs and nasal epithelial cells [25]. We found that RSV infection of primary BECs increased PD-L1-expression robustly, ~30-fold for mRNA and ~4-fold for protein. In contrast rhinovirus-16 up-regulated PD-L1 protein only ~2-fold on human primary BECs [25]. These data, together with the known inhibitory effects of PD-L1 in the context of chronic viral infections [3-5], prompted us to investigate *in vitro* the functional importance of PD-L1 induction in the anti-viral immune responses to RSV infection.

We first hypothesized that un-infected epithelial cells would not activate $CD8^+$ T cells, but in contrast RSV-infected BECs would stimulate $CD8^+$ T cells to express activation markers, proliferate and induce cytokine and cytotoxic protein secretion.

CD69 is one of the earliest activation markers on T cells. The peak proportion of CD69-positive T cells in response to alloantigen stimulation is reported to be <3% [26]. Consistent with this, in our studies, CD69 was present on 2-3% of $CD8^+$ T cells co-cultured with un-infected BECs but this percentage was significantly increased ~3 times on $CD8^+$ T cells co-cultured with RSV-infected BECs. A weaker induction was observed

for CD25 and PD-1 (which is persistently up-regulated on exhausted lymphocytes in chronic viral infections) [3-5]. PD-1 was found in low percentages (<0.5%) on our $CD8^+$T cells as they were isolated from normal donors, and was not significantly increased on total $CD8^+$T cells co-cultured with RSV-infected BECs.

The $CD8^+$ T cells most likely to be found in contact with BEC *in vivo* are the EM and TD subpopulations [16, 18]. We observed that the levels of expression of each of CD69, CD25 and PD-1 were higher in these subpopulations than in total $CD8^+$ T cells co-cultured with un-infected BECs, and their induction by RSV-infected BECs was greater in the EM and TD subpopulations than in total $CD8^+$ T cells. These data suggest the $CD8^+$T cells which respond to RSV-infected BECs by activation are the EM and TD $CD8^+$ T cell subsets.

PHA stimulated-$CD8^+$ T cells did not proliferate in un-infected BEC/$CD8^+$ T cell co-cultures. However, the majority of $CD8^+$ T cells co-cultured with RSV-infected BECs underwent more than one round of proliferation. Polyclonally-stimulated $CD8^+$ T cells cultured alone proliferated in a manner similar to $CD8^+$ T cells co-cultured with RSV-infected BECs, suggesting that un-infected epithelium has actually the capacity to inhibit $CD8^+$ T cell proliferation, similar to results obtained *in vitro* using of murine cells [27].

Cytokine (IFN-γ/IL-2) and cytotoxic protein (granzyme/perforin) production showed similar patterns as for the activation markers, with higher induction occurring in $CD8^+$ T cells co-cultured with RSV-infected BECs compared to un-infected BECs. Induction of IFN-γ in $CD8^+$ T cells was chiefly among the effector cell populations, while IL-2, was also induced but more so among the naïve cells. Granzyme B and perforin production was found almost exclusively in EM and TD subpopulations. However, $CD8^+$ T cells expressed high levels of cytotoxic proteins, especially granzyme B, even when co-cultured with un-infected BECs, thus their increase in numbers was relatively modest.

These data are in accordance with anticipated function, with resting BECs not activating $CD8^+$ T cells, but RSV-infected BECs equipping local $CD8^+$ T cells for appropriate expansion and anti-viral effector function.

We next hypothesised that increased PD-L1 expression in this context might prevent full activation of anti-viral effector functions. Indeed, we found a further increase of IFN-γ-, IL-2- and granzyme B-levels in the presence of PD-L1 (but not PD-L2) blocking

antibody, confirming that increased expression of PD-L1 played an inhibitory role in this system.

There are relatively few models investigating functional memory T cell responses to live virus infections. We therefore developed our *in vitro* co-culture system to allow us to study these events in a system modelling epithelial/$CD8^+$ T cell interactions as they may occur *in vivo*. T cells are found in association with epithelium in the respiratory tract and it has been demonstrated that human bronchial epithelial cells have the capacity to present antigens and directly activate cytotoxic $CD8^+$ T cells [28]. There is substantial evidence that effector-memory $CD8^+$ T cells do not proliferate in the lung or airways, suggesting that there is continual recruitment of new cells from the circulation [19]. Therefore by using $CD8^+$ T cells isolated from peripheral blood we tried to create *in vitro* an environment similar to that found in the human respiratory tract *in vivo*. Our data confirm that PD-L1 can indeed play an inhibitory role in local anti-viral interactions between BECs and $CD8^+$ T cells in acute respiratory infections.

These findings may have important implications in increased susceptibility to RSV infection in infants, as well as in incomplete memory immune responses to RSV in older children, adults and the elderly. Blocking PD-L1 may be an attractive therapeutic approach to augment anti-viral immunity in acute respiratory viral infections such as RSV.

1. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol*. 2005; 23:515-48.
2. Keir ME, Francisco LM, Sharpe AH. PD-1 and its ligands in T-cell immunity. *Curr Opin Immunol*. 2007; 19(3):309-14.
3. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, Mackey EW, Miller JD, Leslie AJ, DePierres C, Mncube Z, Duraiswamy J, Zhu B, Eichbaum Q, Altfeld M, Wherry EJ, Coovadia HM, Goulder PJ, Klenerman P, Ahmed R, Freeman GJ, Walker BD. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature*. 2006; 443(7109):350-4.
4. Penna A, Pilli M, Zerbini A, Orlandini A, Mezzadri S, Sacchelli L, Missale G, Ferrari C. Dysfunction and functional restoration of HCV-specific CD8 responses in chronic hepatitis C virus infection. *Hepatology*. 2007; 45(3):588-601.
5. Boni C, Fisicaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertoletti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol*. 2007; 81(8):4215-25.
6. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, Ahmed R. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*. 2006; 439(7077):682-7.
7. Iwai Y, Terawaki S, Ikegawa M, Okazaki T, Honjo T. PD-1 inhibits antiviral immunity at the effector phase in the liver. *J Exp Med*. 2003; 198(1):39-50.
8. Lafon M, Megret F, Meuth SG, Simon O, Velandia Romero ML, Lafage M, Chen L, Alexopoulou L, Flavell RA, Prehaud C, Wiendl H. Detrimental contribution of the immuno-inhibitor B7-H1 to rabies virus encephalitis. *J Immunol*. 2008; 180(11):7506-15.

9. Hull J, Thomson A, Kwiatkowski D. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. *Thorax*. 2000; 55(12):1023-7.
10. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P. The burden of respiratory syncytial virus infection in young children. *N Engl J Med*. 2009; 360(6):588-98.
11. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med*. 2005; 352(17):1749-59.
12. Schwarze J, O'Donnell DR, Rohwedder A, Openshaw PJ. Latency and persistence of respiratory syncytial virus despite T cell immunity. *Am J Respir Crit Care Med*. 2004; 169(7):801-5.
13. Hegele RG, Hayashi S, Bramley AM, Hogg JC. Persistence of respiratory syncytial virus genome and protein after acute bronchiolitis in guinea pigs. *Chest*. 1994; 105(6):1848-54.
14. Sorkness RL, Gern JE, Grindle KA, Mosser AG, Rosenthal LA, Mikus LD, Lemanske RF, Jr. Persistence of viral RNA in 2 rat strains differing in susceptibility to postbronchiolitis airway dysfunction. *J Allergy Clin Immunol*. 2002; 110(4):607-9.
15. Wilkinson TM, Donaldson GC, Johnston SL, Openshaw PJ, Wedzicha JA. Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med*. 2006; 173(8):871-6.
16. Ely KH, Cauley LS, Roberts AD, Brennan JW, Cookenham T, Woodland DL. Nonspecific recruitment of memory CD8+ T cells to the lung airways during respiratory virus infections. *J Immunol*. 2003; 170(3):1423-9.
17. Ostler T, Hussell T, Surh CD, Openshaw P, Ehl S. Long-term persistence and reactivation of T cell memory in the lung of mice infected with respiratory syncytial virus. *Eur J Immunol*. 2001; 31(9):2574-82.
18. Kohlmeier JE, Woodland DL. Immunity to respiratory viruses. *Annu Rev Immunol*. 2009; 27:61-82.
19. Hikono H, Kohlmeier JE, Ely KH, Scott I, Roberts AD, Blackman MA, Woodland DL. T-cell memory and recall responses to respiratory virus infections. *Immunol Rev*. 2006; 211:119-32.
20. Papi A, Stanciu LA, Papadopoulos NG, Teran LM, Holgate ST, Johnston SL. Rhinovirus infection induces major histocompatibility complex class I and costimulatory molecule upregulation on respiratory epithelial cells. *J Infect Dis*. 2000; 181(5):1780-4.
21. Kim J, Myers AC, Chen L, Pardoll DM, Truong-Tran QA, Lane AP, McDyer JF, Fortuno L, Schleimer RP. Constitutive and inducible expression of b7 family of ligands by human airway epithelial cells. *Am J Respir Cell Mol Biol*. 2005; 33(3):280-9.
22. Stanciu LA, Bellettato CM, Laza-Stanca V, Coyle AJ, Papi A, Johnston SL. Expression of programmed death-1 ligand (PD-L) 1, PD-L2, B7-H3, and inducible costimulator ligand on human respiratory tract epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines. *J Infect Dis*. 2006; 193(3):404-12.
23. Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol*. 2007; 8(3):239-45.
24. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999; 401(6754):708-12.
25. Heinecke L, Proud D, Sanders S, Schleimer RP, Kim J. Induction of B7-H1 and B7-DC expression on airway epithelial cells by the Toll-like receptor 3 agonist double-stranded RNA and human rhinovirus infection: In vivo and in vitro studies. *J Allergy Clin Immunol*. 2008; 121(5):1155-60.
26. Craston R, Koh M, Mc Dermott A, Ray N, Prentice HG, Lowdell MW. Temporal dynamics of CD69 expression on lymphoid cells. *J Immunol Methods*. 1997; 209(1):37-45.
27. Wang H, Su Z, Schwarze J. Healthy but not RSV-infected lung epithelial cells profoundly inhibit T cell activation. *Thorax*. 2009; 64(4):283-90.
28. Kraetzel K, Stoelcker B, Eissner G, Multhoff G, Pfeifer M, Holler E, Schulz C. NKG2D-dependent effector function of bronchial epithelium-activated alloreactive T-cells. *Eur Respir J*. 2008; 32(3):563-70.

CURRICULUM VITAE

(April 2010)

PERSONAL DETAILS:

Name: Aurica Georgeta TELCIAN

DOB: September 9th, 1974

Nationality: Romanian

E-mail: a.telcian@imperial.ac.uk

EDUCATION:

- 1993-1999: Faculty of General Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania – Degree of Physician/ Oct 1st, 1999
- 2000-2006: Specialist training in Laboratory Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania – Certificate of Specialist Physician in the field of Laboratory Medicine/ May 25th, 2006
- 2005-2010: PhD student (part-time), Physiology Department, "Iuliu Hatieganu" University of Medicine and Pharmacy Cluj-Napoca, Romania

WORK EXPERIENCE:

- January 2000-February 2001: Junior doctor, Municipal Hospital of Cluj-Napoca, Romania
- March 2001-October 2002: Resident in Laboratory Medicine, Bacteriology Department, Infectious Disease Hospital, Cluj-Napoca, Romania
- November 2002-June 2003: Resident in Laboratory Medicine, Biochemistry Department, 2nd Internal Medicine Hospital, Cluj-Napoca, Romania
- July 2003-July 2005: Resident in Laboratory Medicine, Hematology Department, "Ion Chiricuta" Cancer Institute, Cluj-Napoca, Romania
- July 2005-August 2008: Academic visitor, Respiratory Medicine Department, Imperial College London, UK

- August 2008-present: Research Assistant, Respiratory Medicine Department, Imperial College London, UK

PUBLICATIONS:

- Aurica G Telcian, Adriana Muresan, Vasile-Laza Stanca, Sebastian L Johnston, Luminita A Stanciu, "$CD8^+$ T cell cytokine & cytotoxic protein production and proliferation is differentially regulated by normal and RSV-infected human bronchial epithelial cells", Clujul Medical, vol 83, no 1:64-68, 2010
- Aurica G Telcian, Adriana Muresan, Vasile-Laza Stanca, Sebastian L Johnston, Luminita A Stanciu, "Gene expression of Programmed Death Ligand (PD-L)-1, PD-L2, Inducible Costimulator Ligand and B7-H3 on human alveolar epithelial cells and regulation by respiratory syncytial virus and type 1 and 2 cytokines", Physiology, vol 19.3 (63):4-7, 2009
- Aurica G Telcian, Vasile-Laza Stanca, Michael R Edwards, James A Harker, Hongwei Wang, Nathan W Bartlett, Patrick Mallia, Mihnea T Zdrenghea, Tatiana Kebadze, Anthony J Coyle, Peter JM Openshaw, Luminita A Stanciu, Sebastian L Johnston, "RSV-induced bronchial epithelial cell PD-L1 expression inhibits $CD8^+$T cell anti-viral activity" [*manuscript in preparation*]
- Zdrenghea MT, Laza-Stanca V, Telcian A, Johnston SL, Stanciu LA. "Respiratory epithelial cell calcitriol metabolism modulation by viruses: putative role in virus-induced asthma exacerbations". J Rom Soc Allergol Clin Immunol 2007;4.
- Popa L, Telcian A.G. "Molecular bases of new strategies in the treatment of cancer", Rev Obstetric Gynecol 1996; 44: 23-26.

ORAL PRESENTATIONS:

- "Calcitriol increases interferon stimulated gene expression and has anti-viral and anti-inflammatory activity in human bronchial epithelial cells", The XXVIII Congress of the EAACI, Warsaw, Poland, 6-10 June 2009

- "Respiratory Syncytial Virus negatively modulates vitamin D action in respiratory epithelial cells", The XXVII Congress of the EAACI, Barcelona, Spain, 7-11 June 2008
- "Presentation of a fellowship experience in the United Kingdom", ERS Congress, Stockholm, Sweden, 15-19 September 2007
- "Functional effect of RSV-induced PD-L1 on epithelial cell activation of $CD8^+$T cells", ATS Congress, San Francisco, US, 18-23 May 2007, awarded with an International Trainee Travel Award
- "RSV modulation of co-stimulatory molecules on primary human bronchial epithelial cells", EAACI-GA2LEN Summer School, Chalkidiki, Greece, 23-27 June 2006
- "Primary extramedullary, pleuropericardial, myeloid tumour. Case report and literature review"; The Jubilee Session of "Ion Chiricuta" Cancer Institute Cluj-Napoca, 6-9 October 2004, Society Journal, p 43

POSTER PRESENTATIONS:

- A.G. Telcian, L.A. Stanciu and S.L. Johnston. "Effector memory $CD8^+$T cell responses to RSV infected respiratory epithelial cells", ERS Congress, Vienna, Austria, 12-16 Sept 2009
- Aurica G Telcian, Luminita A Stanciu and Sebastian L Johnston. "Calcitriol increases interferon stimulated gene expression and has antiviral and anti-inflammatory activity in human bronchial epithelial cells", ATS Congress, San Diego, US, May 2009
- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S.L. Johnston. "The role of RSV infected epithelial cells on $CD8^+$T cell responses", EAACI-GA2LEN Allergy School, Cluj-Napoca, Romania, 5-10 September 2007
- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S.L. Johnston. "$CD8^+$T cell responses to RSV infected respiratory epithelial cells", The XXVI Congress of the EAACI, Goteborg, Sweden, 9-13 June 2007 – awarded with a Junior Poster Prize
- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S. L. Johnston. "Functional effect of RSV-induced PD-L1 on epithelial cell activation of $CD8^+$T cells", The 5^{th} EAACI-

GA2LEN-DAVOS meeting, Basic Immunology on Allergy and Clinical Immunology, Davos, Switzerland, 1-4 February 2007

- AG Telcian, V. Laza-Stanca, L. A. Stanciu and S. L. Johnston. "Expression of PD-L1 on primary human bronchial epithelial cells and its regulation by Respiratory Syncytial Virus, IFN-γ and IL-4", The XXV Congress of the EAACI, Vienna, Austria, 10-14 June 2006 – awarded with a Junior Poster Prize

ABSTRACT COLABORATIONS:

- Sarah Essilfie-Quaye, Andy Durham, Aurica Telcian, Joseph Footitt, Ian Adcock and Sebastian Johnston. "Rhinovirus-induced HDAC2 nitration and reduced HDAC2 activity in THP-1 cells", ERS Congress, Sept 2010 [*submitted*]
- J.Footitt, MB Trujillo-Toralbo, A. Telcian, P. Mallia & SL Johnston. "Changes in FEV1 and lower respiratory tract scores in a human model of rhinovirus induced COPD exacerbations", 2nd International Congress on Exacrebation of Airway Disease, Miami, April 2010 [*submitted*]
- GN Feketea, AG Telcian, MR Edwards, LA Stanciu and SL Johnston. "IL-4 modulation of rhinovirus-induced CCL20/MIP-3a production in bronchial epithelial cells", ERS Congress, Vienna, Austria, 12-16 Sept 2009
- LA Stanciu, N Rushwan, A Telcian, M Zdrenghea, SL Johnston. "Rhinovirus infection differentially modulates MHC class I-related chain (MIC) A and B molecules in human respiratory epithelial cells", ERS Congress, Vienna, Austria, 12-16 Sept 2009
- G Feketea, AG Telcian, LA Stanciu, SL Johnston. "CCL20 production in bronchial epithelial cells is increased by rhinovirus infection" The XXVIII Congress of the EAACI, Warsaw, Poland, 6-10 June 2009 [oral presentation]
- LA Stanciu, AG Telcian, MT Zdrenghea, V Laza-Stanca, SL Johnston. "The effect of calcitriol on VDR, RANTES and virus gene expression in rhinovirus-infected respiratory bronchial epithelial cells" The XXVII Congress of the EAACI, Barcelona, Spain, 7-11 June 2008
- Luminita A. Stanciu, Cinzia M. Bellettato, Mihnea T. Zdrenghea, Vasile Laza-Stanca, Aurica G.Telcian, et al. "RSV modulation of co-stimulatory molecules on

human respiratory epithelial cells", American Association of Immunologists Meeting, Boston, May 2006

AWARDS:

- Scholarship Award from the UCB Institute of Allergy (session of April 2005) for 6 months at Imperial College London, Respiratory Medicine
- Long Term Research ERS Fellowship (May 2006) for 1 year at Imperial College London, Respiratory Medicine
- HC Roscoe (2009) Grant – British Medical Association, Dr Aurica Telcian - Imperial College London, "Virus infection of epithelial cells modulates molecules regulating cytotoxity: role in asthma exacerbations"

FOREIGN LANGUAGES: English, French: written/spoken