PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
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LUIZ CARLOS RODRIGUES JUNIOR

OTIMIZAÇÃO DA RESPOSTA DE CÉLULAS T CD8+ DE MEMÓRIA AO HERPES SIMPLEX VIRUS-1 UTILIZANDO TERAPIA GENÉTICA COM INTERLEUCINA-15 E INTERLEUCINA-21

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ORIENTADADOR: Dra. CRISTINA BONORINO

CO-ORIENTADOR: Dr. BARRY T. ROUSE

PORTO ALEGRE

SETEMBRO DE 2008

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BANCA EXAMINADORA

_____

Dr. Carlos Alexandre Sanches Ferreira - PUCRS

_____

Dra. Denise Cantarelli Machado - PUCRS

_____

Dr. Paulo Michel Roehe - UFRGS

_____

Dr. Aguinaldo Pinto - UFSC

DEDICO ESSE TRABALHO AOS MEUS PAIS E A QUALQUER COISA OU FORÇA DESCONHECIDA QUE TENHA ME AJUDADO, SEJA ELA QUAL FOR.

RESUMO

Herpes Simplex Vírus-1 (HSV-1) é um membro da família *Herpesviridae* e subfamília *alfa-herpesvirinea* bastante disseminado entre os seres humanos. Esse vírus inicia seu processo de infecção a partir das células epiteliais da superfície da pele e mucosas, atingindo o sistema nervoso periférico. O HSV-1 inicia a infecção através da mucosa oral e fica localizado na forma latente no nervo trigêmio da face, algumas vezes, pela ação de fatores endógenos ou exógenos, esse vírus volta a sua forma ativa, ocasionando a reincidiva . Nesse processo de reativação do vírus ele pode se localizar, na mucosa oral (gengivoestomatite herpética), no nervo óptico (queratite herpética) e no sistema nervoso central (encefalite). No caso da gestante, a reativação herpética pode ser assintomática, o que pode infectar o filho no momento do parto, levando a danos neurológicos irreversíveis ou a morte. O processo de latência é coordenado por dois mecanismos principais, a expressão dos genes LAT e a resposta imunológica. As células da resposta imune que bloqueiam a reativação viral a partir do nervo são os linfócitos T CD8+ de memória. Esses linfócitos ficam justapostos à membrana do corpo celular do neurônio, interagindo com o epítopo SSIEFARL de uma glicopoteína gB do envelope viral. Os linfócitos T CD8+ SSIEFARL específicos produzem citocinas, como IFN-γ, que penetram no neurônio e impedem a expressão de genes que estão envolvidos na construção do capsídeo, determinantes para formação de novos vírions. Quando o número dessas células diminui, o vírus volta a sua forma ativa. A proliferação e função das células T CD8+ é controlada por citocinas, principalmente a IL-15 e a IL-21. Muitos estudos indicam que elas têm um papel na proliferação homeostática de células T CD8+. Nesse trabalho, foram elaboradas construções gênicas com o DNA da IL-15 e da IL-21 para avaliar o potencial dessas citocinas na otimização da resposta T CD8+ de memória ao HSV-1. *In vitro*, a IL-21 aumentou a freqüência de células T CD8+, com ou sem estimulação de TCR. Na fase efetora, a IL-15 e IL-21 aumentaram os números de células T CD8+ antígeno-específicas produtoras de IFN-γ. Para os estudos de memória foi utilizado um sistema de transferência de células SSIEFARL transgênicas de camundongos CD90.2 doadores para camundongos CD90.1 receptores. Os camundongos receptores foram infectados com HSV-1 pela rota intraperitoneal e tratados com o plasmídeo contendo de cada citocina ou a combinação deles, um plasmídeo que codificava a glicoproteína gB do HSV-1 foi utilizado com fonte de antígeno Os resultados mostraram que cada pIL-15 ou pIL-21 isoladamente induz a proliferação de células T CD8+ de memória ao herpes e que a administração do antígeno não teve grande influência. Por outro lado, a combinação de pIL-15, pIL-21 e pgB foi mais eficiente, tanto no aumento dos números de células T CD8+, quanto na expressão de IFN-γ.

ABSTRACT

Herpes Simplex Vírus-1 (HSV-1) is a member of *Herpesrviridae* family and *alpha-herpesvirinea* subfamily widely spread among human beings. This virus begins the infection through epithelial cells from skin and mucosal surface reaching the peripheral nervous system. HSV-1 infects the oral mucosa and establishes latency in trigeminal ganglia. Sometimes by action of endogenous or exogenous factors these viruses returns to active form leading to viral reactivation. During this process the virus can relocalizes on oral mucosal (hepetial genvivostomatites), optical nerve (ocular keratites) and central nervous system (encephalitis). In case of pregnancy, the reactivation could be asymptomatic; it could be dangerous because the virus can infect the baby during the childbirth, leading to neurological conditions and sometimes death. The process of latency is governed by latency associated transcripts (LAT genes) an immunological response as T cells. The cells that block the viruses from reactivation from the nerve are the memory $CD8^+$ T cells. These lymphocytes stay adsorbed to neuron membrane interacting with the epitope SSIEFARL from a gB glycoprotein of HSV-1 envelop.. SSIEFARL $CD8^+$ T lymphocytes produces cytokines as IFN-γ that penetrates into neuron and blocks the expression of genes involved in virion assembly and formation. When the number of $CD8^+$ T cells reduces the viruses returns to active form. The proliferation and function of CD8+ T cells is controlled by cytokines, mainly IL-15 and IL-21. Several studies have shown that this cytokines have crucial a role in homeostatic proliferation of CD8+ T cells. In this study plasmid coding to IL-15 and IL-21 were elaborated to investigate the role of these cytokine on HSV-1 memory CD8+ T cell proliferation. *In vitro*, pIL-21 increased the frequency of CD8+ T cells in presence or absence of TCR stimulation. When administered during effector phase of HSV-1 infection pIL-15 and pIL-21 increased the numbers of antigen specific CD8+ T cells that produce IFN-γ. For memory studies an adoptive transfer system was applied. SSIEFARL transgenic cells from $CD90.2^+$ donor mice were transferred to $CD90.1^+$ holster mice. The $CD90.1^+$ holster was infected with HSV-1 intraperitoneally and tread with each cytokine plasmid or combination, gB coding plasmid was used as antigen source. The resulted showed the pIL-15 or pIL-21 alone can induce proliferation of HSV-1 memory CD8+ T cells and that antigen did not have significant influence when provide together with the cytokines plasmids. However, the combination of pIL-15, pIL-21 and pgB together was more efficient to cell numbers and IFN-γ production.

LISTA DE ABREVIATURAS

Bcl-2: B Cell Leukemia 2 (molécula antiapoptótica)
BcL-xL: *B cell leukemia/lymphoma-xL* (molécula anti-apoptótica)
Bim: BcL-2 *Interacting mediator of cell death* (molécula apoptótica)
CCR7: receptor de quimiocina
DC: Célula Dendrítica
CD62L: L-selectina
CMV: Citomegalovirus
CpG: dinucleotídeo Citosina/Guanosina
DNA: Ácido desoxirribonucléico
dDC: Células dendríticas dermais
EBV: Epstein-Baar Vírus
gB: glicoproteína B
gC: glicoproteína C
gD: glicoproteína D
gH: glicoproteína H
gL: glicoproteína L
HSV: Herpes Simplex Vírus
HS: Sulfato de Heparano
HIV: Vírus da Imunodeficiência Humana
HSK; Queratite por HSV-1
HHV-6: Herpes Virus humano- 6A
HHV-7: Herpes Virus Humano 7
HLA: Antígeno Leucocitário humano
HEVEM: Mediador da entrada de Herpes virus
IFN: Interferon
IgE: Imunoglobulina E
IgG: Imunoglobulina G
IL: Interleucina
KSHV: Herpes virus associado a sarcoma de kaposi
LC: Células de Langerhans
LAT: Transcritos Associados a Latência
MHC: Complexo Principal de Histocompatibilidade
NK: *Natural Killer*
PKR: serina/treonina quinase
RNA: acido ribonucleico
T_H1: T auxiliar 1
T_H2: T auxiliar 2
TLR: *Toll Like Receptor*
TCR: Receptor de Célula T
Tγδ: Linfóctio T gama delta
VZV: Varicella Zoster Virus

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INTRODUÇÃO

Os vírus da família *Herpesviridae* são altamente disseminados na natureza, sendo encontrados tanto em homens quanto em animais (MACKIE, 2003). Eles são constituídos por um DNA de fita dupla envolto por um capsídeo protêico e externamente apresentam um envelope de constituição lipídica (OIEN et al, 1997). A família herpesviridae apresenta três subfamílias: alfa-herpesvirinae, beta-herpesvirinae e gama-herpesvirinae. Os alfa-herpesvírus são representados pelo HSV-1 e pelo HSV-2, que causam infecções orais e genitais, respectivamente. Também está dentro dessa subfamília o VZV, que causa infecções na pele. Na subfamília dos beta-herpesvírus estão CMV, HHV-6 e HHV-7. Esses geralmente causam infecção em múltiplos órgãos. Na família dos gama-herpesvírus, encontra-se o EBV, que infecta células B, que normalmente causa uma infecção branda e com poucos sintomas, e o KSHV (HELDWEIN et al, 2008).

Embora estruturalmente e geneticamente similares, o HSV-1 e o HSV-2 diferenciam-se basicamente pela forma de transmissão e o sítio da lesão produzida. O HSV-1 geralmente ataca a mucosa oral, enquando o HSV-2 usualmente ataca a mucosa genital entretanto, não existe uma afinidade de ligação específica para cada um deles nesses sítios (WAGGONER-FOUNTAIN et al, 2004). A ampla prevalência desse vírus pode ser atribuída a muitos fatores, tais como a facilidade de transmissão, a possibilidade de infectar indivíduos de qualquer faixa etária independente de sexo, a manutenção do estado de latência viral, criando o subseqüente portador de doença ativa assintomática, os mecanismos de escape do vírus ao sistema imune, além da própria resistência do vírus (WAGGONER-FOUNTAIN et al, 2004; SACKS et al, 2004, WEISS, 2004). Estudos epidemiológicos atuais indicam também que a associação bi-direcional entre o HSV e o HIV aumenta o risco de aquisição do HIV e a expressão clínica do HVS (FREEMAN et al, 2006). Entretanto, ambos, HSV-1 e HSV-2, não apresentam uma elevada morbidade para indivíduos jovens e adultos com o sistema

imune competente, podendo ser, por outro lado, extremamente letais quando infectam neonatos, idosos e pacientes com alguma deficiência imune (WAGGONER-FOUNTAIN et al, 2004; MORFIN et al, 2003). De acordo com o "status" imunológico, os indivíduos infectados podem desenvolver quadros clínicos como uma lesão circunscrita a uma região da pele e lábios, a gingivoestomatite herpética primária (ARDUINO et al, 2008), infecção ocular herpérica (queratite hepética) (LEPISTO et al, 2007), encefalite e herpes disseminado envolvendo múltiplos órgãos (SIMMONS, 2002). A HSK é considerada a segunda maior causa de perda de visão decorrente de infecção, e a maioria dos indivíduos que desenvolvem HSK tem história de herpes labial (SACKS et al, 2004).

A infecção em neonatos pode acontecer em cerca 33% a 50% daqueles nascidos de mulheres com herpes ativo recorrente e que muitas vezes não apresentam sintomas. A infecção acontece no momento do parto. Essas crianças, quando desenvolvem encefalite, apresentam uma sobrevida alta, embora à maioria desenvolva seqüelas neurológicas, quando desenvolvem doença disseminada, em torno de 50% morrem, mesmo sob terapia antiviral (BROWN, 1998)

A infecção por HSV-1 ou HSV-2 ainda não tem cura e a terapêutica aplicada durante os períodos de infecção assintomática é realizada com medicamentos antivirais. O antiviral mais utilizado é o Aciclovir, um análogo de guanosina que inibe a replicação viral. Este fármaco funciona bem em pacientes imunocompetentes, sendo administrado topicamente ou sistemicamente, dependendo do caso (SWEARINGEN et al, 2007). No caso dos neonatos e imunocomprometidos, esse tratamento requer uma terapêutica mais intensiva e que as vezes não funciona, além de levar ao desenvolvimento de cepas resistentes ao Aciclovir (LEVIN et al, 2004). Tem sido estudado também a utilização de anti-CD3 para o tratamento do HSK, ele reduz a infiltração de células inflamatórias e CD4+ na córnea, diminuindo a severidade da lesão, entretanto esses estudos ainda estão em modelos animais (SARANGI et al, 2008).

Diante desse quadro, a infecção por HSV é considerada um problema de saúde pública mundial e o seu controle e tratamento são muitas vezes difíceis. Atualmente, tem-se investido muito na busca de vacinas para uso profilático para prevenir a infecção primária, assim como terapias, para os casos de infecção recorrente (JONES et al, 2004).

Várias formas de vacinas anti-virais são investigadas utilizando o vírus inativado ou atenuado, componentes da estrutura do vírion, produção de proteínas virais recombinantes, cepas com replicação descontínua e o próprio DNA viral (KOELLE et al, 2003). Alguns componentes da estrutura do vírion, capazes de direcionar a resposta imune, estão sendo produzidos como peptídeos sintéticos e testados em diferentes sistemas de imunização. Os mais bem caracterizados são as glicoproteínas do envelope $gB_{498-505}$ (SSIEFARL) e gD_{1-23} (ADASLKMADPNRFRGKDLPR), reconhecidos como epítopos que geram resposta citotóxica e humoral em camundongos, respectivamente (MESTER et al, 1990; YU et al, 1998). Esses epítopos têm sido investigados para o desenvolvimento de uma vacina anti-HSV, e têm apresentado resultados significativos quanto ao aumento da resposta imune anti-viral, principalmente quando aplicados na forma de vacina de DNA (LEE et al, 2003).

Entretanto, as vacinas e terapias atuais construídas baseadas nesses epítopos do HSV não têm apresentado resultados suficientemente satisfatórios do ponto de vista de proteção, geração de células $CD8^+$ de memória com conseqüente prevenção da reativação. Uma vacina eficiente para prevenir a infecção por HSV deve proporcionar uma potente resposta imune celular, principalmente mediada por $CD8^+$, e humoral, para que o vírus não ganhe acesso aos neurônios. Por outro lado, para impedir a reativação viral a partir do gânglio neuronal, a indução da proliferação e otimização da função das células T CD8+ de memória deve ser priorizada (SHERIDAM et al, 2003). Uma terapia eficiente deve induzir a manutenção de quantidades de células T $CD8^+$ de memória gB específicas suficientemente capazes de bloquear a reativação viral no indivíduo (KOELLE et al, 2008). Entretanto, esse padrão de

resposta não é fácil de ser obtido, tendo em vista que a eficiência da resposta $CD8^+$ de memória depende da montagem da resposta inata e de como estas células de memória serão mantidas após a infecção primária (BEHBOUDI et al, 2004; MASSON et al, 2008). Algumas citocinas como a IL-15 e a IL-21 foram identificadas como cruciais no processo de ativação e manutenção das células $CD8^+$ (LI et al, 2005, MOROZ et al, 2004).

Alternativas para melhorar a resposta ao HSV envolvem "manobras" para aprimorar a programação da célula T $CD8^+$ durante a resposta inata e também para que um maior número de células sobreviva à fase de declínio após a retirada do vírus, e que se tornarão células de memória efetoras (GIERYNSKA et al, 2002). Além disso, o modelo utilizado para melhorar a resposta deve contribuir também para manutenção da proliferação e na atividade funcional das células T $CD8^+$ de memória (OSORIO et al, 2005, TOKA et al, 2005).

Dentro desse contexto, uma alternativa são ILs. As ILs são moléculas mediadoras de respostas celular, tanto na fase inata quanto na adaptativa. Existe uma variedade de tipos ILs, mas a principal divisão se estabelece entre ILs de padrão inflamatório (T_H1) e anti-inflamatório (T_H2) (ONOÉ et al, 2007). Entretanto, outras subdivisões já foram estabelecidas, proncipalmente após o sequenciamento do genoma humano. Existem citocinas que utilizam o receptor gama (γ), como IL-2, IL-7, IL-15 por exemplo, que induzem proliferação; citocinas que que utilizam o receptor alfa (α), como a IL-28 A, IL-28B e IL-29 e citocinas que atuam preferencialmente em respostas autoimunes, como a IL-17 (T_H3) (STEINKE et al, 2006). A resposta imune ao HSV é regulada por citocinas e as mais investigadas são a IL-2, IL-7, IL-12, IL-15, IL-18 e IL-21 (WIRYANA et al, 1997, CUI et al, 2005, READING et al, 2007) . A IL-15 é responsável pela sobrevivência de células T $CD8^+$ na fase primária efetora (WALLACE et al, 2006), proliferação de células NK (GILL et al, 2005) e proliferação de células T $CD8^+$ de memória (SANDAU, et al, 2007). Ela atua também na função de DCs (DUBSKY et al, 2007). A recentemente descoberta IL-21 atua principalmente na regulação da

maturação e na função das células NK, o que melhora a resposta anti-viral primária (BRADY et al, 2007), e também na proliferação e função de células T $CD8^+$, principalmente quando combinada com a IL-15 (BOLESTA, et al, 2005).

2 – Infeccão e Imunidade ao Herpes Simplex Vírus-1

2.1 Ligação, Fusão e Entrada

A infecção por HSV-1 inicia principalmente pela entrada direta do vírus através da pele ou mucosa, onde ele encontra uma célula alvo para ligação e entrada. Estruturalmente, o HSV-1 apresenta o vírion composto de um capsídeo de formato icosaédrico que envolve e protege o material genético viral, um tegumento e uma membrana externa de natureza lipoproteica com glicoproteínas específicas (gB, gC, gD e gH-gL) (figura 1) (CAIRNS, et al, 2003). Assim que encontra uma célula epitelial, o vírus estabelece uma ligação entre gC e/ou gB e um receptor na superfície da célula. O receptor de ligação para gB é o o HS, um tipo de glicosaminoglicano presente na matriz extracelular e tipos celulares tais como células epiteliais, fibroblastos e células dendríticas (O'DONNELL et al, 2006). Uma vez adsorvido, o HSV-1 necessita ser internalizado pela célula, processo que acontece pela ligação de gD ao receptor de entrada. A gD é específica para os alfa-herpesvírus, essencial para a fusão do envelope com a membrana (HELDWEIN et al, 2008). Como essa proteína não apresenta estrutura de uma proteína de fusão, ela necessita ser ativada no momento da ligação com o receptor (LIGAS et al, 1988). Os receptores de fusão e entrada podem ser de três tipos, de acordo com a célula, HVEM, nectina-1, nectina-2 ou sítios específicos no HS gerados pela ação de 3-O-sulfotransferases. HSV-1 e HSV-2 diferenciam-se algumas vezes quanto à afinidade a diferentes receptores de entrada, por exemplo, nectina-2 não tem ação na entrada do HSV-2 (TAYLOR, et al 2007). HVEM também é expresso em uma variedade de células,

mas não em neurônios, sendo que nesses a entrada é mediada por nectinas (MANOJ et al, 2004). A glicoproteína gL é necessária para a expressão de gH na superfície do envelope, participando do processo de fusão (HELDWEIN et al, 2008). Um esquema da organização desses receptores e sua ligação as glicoproteínas virais podem ser observada na figura 1B. São encontradas também, por parte da célula, as regiões com receptores virais associados, as "lipid-raft". No caso do HSV-1 são regiões na membrana da célula hospedeira ricas em colesterol e receptores para gB (BENDER, et al, 2003).

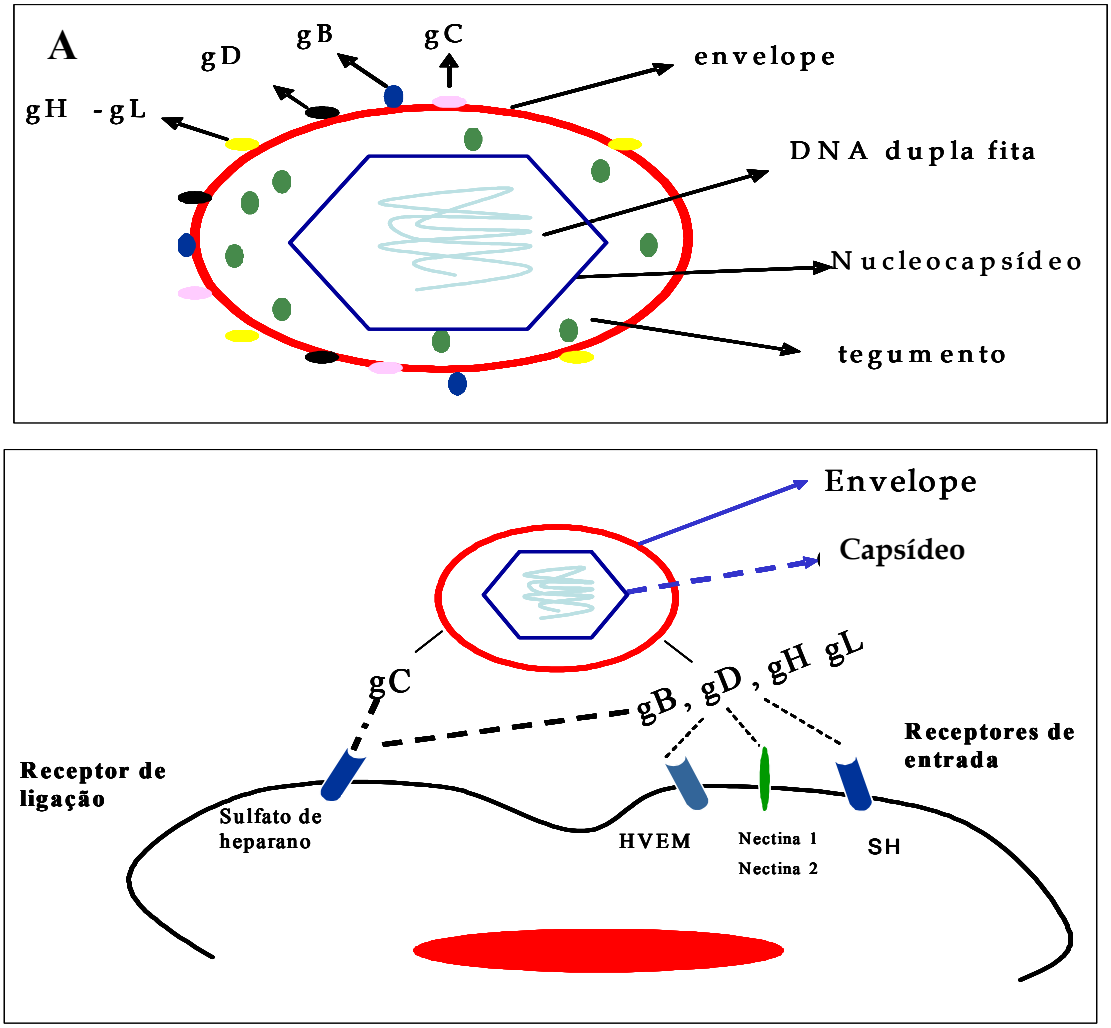

Figura 1: Receptores de superfície celulares e ligantes que participam na adosorção do HSV. **A**: Esquema da estrutura do vírion do HSV-1 representando o material genético, o nucleocapsídeo, o tegumento e as glicoproteínas de envelope. **B)** Interação de receptores e glicoproteínas de ligação de entrada do HSV-1 na célula hospedeira.

Quando dentro da célula as proteínas virais ganham acesso à rota de apresentação de antígenos via MHC de classe I, sem a necessidade de uma nova síntese. O capsídeo chega ao núcleo e o genoma viral inicia um processo de expressão ordenada: genes α ou imediatos, reguladores da replicação, genes β ou primários, envolvidos na síntese de genes tardios e empacotamento do DNA e genes γ ou tardios, envolvidos na síntese de proteínas estruturais do virion (WARD et al, 1994). Proteínas sintetizadas por esses genes podem ser a ICP24, ICP27 e ICP47 nos α, e ICP8 nos β e proteínas de montagem e brotamento nos γ (KOELLE et al, 2003).

Uma característica exclusiva dos alfa-herpesvírus em relação aos membros da família dos herpesvírus é o tropismo desses por células nervosas. No processo de infecção primária, o vírus propaga-se célula a célula a partir da epiderme até a terminação nervosa sensorial mais próxima, aí ele liga-se e entra no neurônio via receptores HS e nectina-1 no HSV-1 e nectina 2 no HSV-2 (DE REGGE et al, 2006). Neurônios são células longas, e o HSV-1 necessita cruzar o axônio para chegar ao núcleo no corpo celular, esse processo é facilitado por proteínas associadas a microtúbulos, responsáveis pelo transporte retrógrato, as dineínas. Por outro lado, quando o vírus necessita sair das células, no processo de reativação, outras proteínas associadas a microtúbulos, as quinesinas, auxiliam o processo anterógrato (DIEFENBACH et al, 2008). Os neurônios tornam-se infectados de maneira produtiva, pois o vírus é transmitido pelas fendas sinápticas. Uma vez localizado no glânglio sensorial o vírus entra em processo de latência, no qual ele pára ou reduz a um nível mínimo a expressão de suas proteínas. Essa latência viral pode ocorrer no glânglio trigêmio, cervical ou lombosacral, de acordo com o ponto de entrada do vírus (MARGOLIS et al, 2007).

2.2 Resposta Imune Primária

No momento da entrada do vírus através da pele ou da mucosa ele encontra, além das células epiteliais, as DCs da pele denominadas LC (Langherin^{+}, CD11c^{+}CD205^{high}CD8α^{low}) e

células dDC (Langherin-, $CD11c^{+}CD205^{low}$ $CD8\alpha^{low}$). Essas DCs são capazes de fazer a fagocitose do vírus mediada por fagossomos, no qual o vírus é destruído e apresentado como um antígeno exógeno (NOVAK et al, 2005). Essas células são consideradas células apresentadoras de antígeno especializadas tendo um papel crítico na aquisição de antígenos, processamento e apresentação de epítopos antigênicos no contexto das moléculas do MHC de classe I ou MHC de classe II. Elas atuam como sentinelas do organismo, determinando o tipo de resposta imune que será gerada para combater o patógeno (STEINMAN, 2001). Os sinais disparados pelos microrganismos nas DCs são mediados por receptores na superfície, esses receptores são denominados de TLR. Existem TLRs específicos para o peptideoglicano (TLR2), para dupla fita de RNA (TLR3), para lipopolissacarídeos (TLR4), flagelina (TLR5), fita simples de RNA (TLR7) e para seqüências CpG de DNA não metilado (TLR9) (PASARE, et al, 2004). Estudos recentes têm demonstrado que em modelo murino de infecção da mucosa genital com HSV-2, ocorre a indução da secreção de IFN tipo I (α/β) por pDC ($CD11b^{+}CD11c^{+}CD8\alpha^{low}$) e que essa indução é mediada pela ligação do CpG do DNA viral ao TLR9 (LUND et al, 2003; KODAWAKI et al, 2002). As pDCs são capazes de produzir altas quantidades de IFN tipo I em resposta a vírus e CpGs de DNA, estabelecendo uma ligação entre a resposta inata e adaptativa (KODAWAKI et al, 2002). O papel determinante das pDCs e sua ativação via TLR-9 como primeira linha de produção de IFN-α na infecção herpética ficou mais evidente quando se comparou camundongos desprovidos de pDC e com o gene do TLR-9 truncado. Observou-se que eles apresentam as mesmas características clínicas, histológicas e padrão de sobrevivência muito similar. Além disso, as pDCS estão presentes no sítio antes da infecção, e mais células são recrutadas após a infecção (JENNIFER, 2006). Entretanto, essas células originam apenas a primeira onda de IFN tipo I, no decorrer da infecção outras células que se tornam responsáveis pela produção desse IFN, por mecanismos independentes de TLR-9 (RASMUSSEN et al, 2007)

A maioria das LC e dDC, no momento que encontra o vírus passa a ser infectada por ele. Isso é possível porque as DCs apresentam receptores de ligação e entrada para o vírus. Uma vez que o vírus entra na célula, mediado pela ligação aos receptores HS e HEVM, ele não é mais apresentado como um antígeno, mas passa a funcionar com um agente infectante da DC (BOSNJAK et al, 2005). O HSV-1 infecta, principalmente, DCs em um estágio imaturo e acaba por promover a maturação fenotípica parcial dessas células. Entretanto, essa maturação é refratária e temporária, pois após esse período inicial as células param de maturar, ocorre o bloqueio da expressão de IL-12, e tornam-se incapazes de induzir a estimulação e proliferação de células T específicas, além de tornarem-se apoptóticas (POLLARA et al, 2003).

Nessa situação, em que as primeiras DCs que entraram em contato com o vírus tornam-se infectadas por ele, poderia se dizer que a resposta imune inata falhou. Entretanto, o sistema imune lança uma alternativa para impedir a infecção viral. Uma outra sub-população de DCs ($CD11c^+$ $CD8\alpha^+$), não infectadas pelo vírus e que também estão em um estágio de diferenciação imaturo, mas acabam maturando por ação da liberação direta de IFN tipo I produzido pelas DCs infectadas, promovem a fagocitose das DCs infectadas. As DCs $CD8\alpha^+$ ficam então responsáveis pela apresentação dos antígenos virais, ativando as células T $CD4^+$, um fenômeno conhecido como "apresentação-cruzada" (BOSNJAK et al, 2005; POLLARA et al, 2003)

Outro grupo de células importantes na resposta inata ao HSV são as células NK. Essas células podem fazer parte tanto resposta efetora contra o HSV, sendo ativadas pelo IFN-α/β liberado pelas células infectadas, e promovendo a destruição dessas, quanto na resposta indutora, produzindo IFN-γ como resultado da ação da IL-12 produzida pela célula infectada, ou apenas como resultado do processo de infecção (BIRON et al, 2001). As células NK são particularmente importantes na resposta inata ao HSV porque as células infectadas pelo vírus

apresentam redução nos níveis de expressão do MHC de classe I, o que desencadeia sinais na NK para promover a sua lise (PIETRA et al, 2000). Além disso, as células NK são uma importante fonte de IFN-γ, uma citocina mediadora da resposta antiviral e que está envolvida na inibição da transcrição do RNA viral; indução de enzimas antivirais como a PKR, induzida por IFN; indução de óxido nítrico (NO); além do recrutamento de células da resposta imune para a região infectada e direcionamento da resposta T_H1 (CHESLER et al, 2002). Um estudo recente identificou um importante mecanismo de ativação das células NK no início de uma resposta viral ou bacteriana. Esse estudo mostrou que nas primeiras horas após uma infecção, as células NK migram para os órgãos linfóides secundários (baço e linfonodos) para se tornarem células NK efetoras pela ação da IL-15 produzida por cDCs, que foram estimuladas por ativação dos ligantes de TLR liberados da infecção. Esse passo é crucial para que a NK deixe o linfonodo pronta para executar as atividades efetoras (LUCAS et al, 2007).

Assim, a resposta primária é composta principalmente pela ação das DCs e das células NK, com seus mecanismos efetores. Entretanto, o vírus escapa a essa resposta inicial e chega aos neurônios, onde essas células não podem atuar. Nesse estágio, inicia a ação da resposta adaptativa, caracterizada por linfócitos B, T $CD4^+$ e T $CD8^+$. Um importante ponto na resposta imune inata é o fato de que, embora o vírus tenha ultrapassado essa "barreira", a ação das células primárias determina a eficiência da resposta adaptativa. Aliás, Bevan e colaboradores demonstraram que a programação eficiente das células T $CD8^+$ de memória depende de um tempo mínimo de sete horas de sinapse entre MHC I da DC e o TCR da CD8 na primeira interação entre essa células (PRLIC et al, 2006). No caso específico da infecção herpética (HSV-1) a qualidade e a quantidade de linfócitos T CD8+ citotóxicos gerados em animais que são deficientes de células NK é extremamente reduzida (NANDAKUMAR et al, 2008).

2.3- Resposta de Memória

No caso das infecções virais, especificamente o HSV-1, embora a reposta inata seja importante nos momentos iniciais, a resposta adaptativa é que estabelece o controle e/ou a eliminação do vírus do organismo (ROUSE et al, 2002). Os linfócitos T CD4 $^{+}$ são os primeiros a dispararem a resposta adaptativa, a importância deles fica mais restrita a montagem da resposta inicial ao HSV-1, sendo pouco significativa durante a latência viral (HARANDI et al, 2001). No início da infecção, mais precisamente durante o processo de entrada, o HSV-1 reduz a sinalização via TCR pela redução dos níveis da fosforilação interna, um processo mediado pelas glicoproteínas de fusão e de entrada. Esse seria um mecanismo do vírus para bloquear as funções efetoras iniciais das células T $CD4^{+}$ (SLOAN et al, 2006). Por outro lado, durante a reativação do vírus, as células T $CD4^{+}$ antígeno específicas têm um importante papel na resolução da lesão (HARANDI et al, 2001).

As células que têm uma importância fundamental no controle da latência e da reativação do HSV-1 são os linfócitos T $CD8^{+}$ (CARBONE et al, 2003). Esses linfócitos são capazes de promover a lise de células infectadas pelo HSV-1, são importante fonte de IFN-γ, atuando tanto na resposta primária efetora contra o HSV-1 quanto na resposta de memória (ROUSE et al, 2002). Eles também contribuem para a manutenção do estado de latência viral dentro do neurônio (KHANNA, 2003).

A latência do HVS-1 ocorre nos neurônios dos gânglios, onde ele fica por toda a vida do indivíduo. Os mecanismos de expressão gênica durante a latência ainda são um pouco controversos. Estudos mais antigos, mas que ainda são relevantes, defendem que não há expressão de antígenos virais durante a latência, e que somente alguns genes que não formam estruturas do vírion e não são antigênicos, conhecidos como LAT, , mas que controlam o processo de latência, são produzidos (KENT et al, 2003; WANG et al, 2005). Já outros estudos mais recentes, principalmente os que envolvem a resposta de linfócitos T $CD8^{+}$,

indicam que algumas genes de glicoproteínas virias, como a gB apresentam níveis replicativos baixíssimos (KHANNA, 2003).

A reativação viral é um evento que pode ou não acontecer após a infecção primária. Ela depende de fatores do hospedeiro e da cepa de vírus, algumas vezes, ele se apresenta de forma assintomática (KNAUP, et al 2000). Para que o vírus volte a sua foram ativa, o vírion deve realizar a rota anterograda a partir do corpo celular pelo axônio e chegar aos dendritos terminais, e voltar a produzir lesão na superfície da pele (JONES et al,2003; BEARER et al, 2000). Nos estudos de Handricks e colaboradores, foi observado que 8 dias após a infecção, no glânglio trigêmio de camundongos infectados com HSV-1, havia uma alta densidade de linfócitos T $CD8^+$ justapostos a membrana, mantendo-se em números elevados por até 84 dias após a infecção primária. Essas células são específicas para o peptídeo gB do HSV e produtoras de IFN- γ (KHANNA, 2003). Posteriormente, observou-se também que na ausência dessas células, em modelos de depleção de linfócitos $CD8^+$, a incidência de encefalite e morte aumentava significativamente. (LANG et al, 2005). Assim, os linfócitos T $CD8^+$ são determinantes na latência e controle da reativação do HVS. Quanto ao fenótipo dessas células, foi observado que elas expressam altos níveis do marcador de superfície celular CD44, o que as caracteriza como linfócitos T $CD8^+$ de memória (KHANNA, 2003).

A hipótese mais estudada para esse mecanismo é a de que o controle da latência do HSV-1 pelas células T $CD8^+$ ocorra pela ação do IFN-γ (PRABHAKARAN, et al 2005). Na latência do HSV-1 é observado um baixíssimo nível de expressão de proteínas (SAWTELL, 2003). Como mencionado anteriormente, os genes dessas proteínas virais são divididos de acordo com a expressão, em imediatos (α), primários (β) e tardios (γ). Os genes γ são subdivididos em γ1, expressos em baixos níveis antes da expressão do DNA viral e γ2, expressos somente após o início da síntese do DNA viral (KOSZ-WNENCHAK, 1993). As células T $CD8^+$ de memória encontradas no glânglio trigêmio apresentam TCR específico

para gB. O mecanismo mais coerente é de que os linfócitos T CD8+, específicos para o gB, reconhecem essa proteína associada ao MHC de classe I, não destroem o neurônio, mas produzem IFN-γ que entra no neurônio infectado e bloqueia a síntese de genes γ2 (KHANNA, 2003, LANG et al, 2005). Mais precisamente, reativação significa montagem do vírion, e o IFN-γ bloqueia a atividade do promotor de ICP0 (gene α), necessário para a reativação, e gC (γ2), que faz parte da montagem da estrutura (DECMAN et al, 2005).

Primeiramente, no início da infecção, os linfócitos T $CD8^+$ reconhecem os epítopos virais apresentados pelas DCs associados ao MHC classe I e a sua ativação e proliferação é dependente de um sinal prévio fornecido pelo linfócito T $CD4^+$ à DC, conhecido como "licensing" (SMITH et al, 2004). Após, os linfócitos T $CD8^+$ entram em fase de expansão, aumentando o número de células efetoras e com grande capacidade de produção de IFN- γ, que dura em torno de cinco dias, o prazo de replicação viral. O pico da resposta acontece no 8º dia, a partir do qual elas entram em fase de contração até o 34º dia, onde permanece um pequeno número de células com o fenótipo de memória ($CD8^+$ $CD44^{high}$). Existem duas classes de linfócitos $CD8^+$ de memória, linfócitos de memória central, que permanecem em órgãos linfóides como os linfonodos, expressando moléculas que direcionam as células para esses órgãos, como CD62L e CCR7 e linfócitos de memória efetora, que produzem IFN- γ e circulam pela região periférica do organismo, não expressando CCR7 e baixos níveis de CD62L (VITETTA et al, 1991). Os linfócitos encontrados no gânglio trigêmio são sub-populações de células efetoras de memória.

Quando os linfócitos T $CD8^+$ entram na fase de contração, o seu número cai drasticamente. Isso foi observado em humanos quando linfócitos de indivíduos soropositivos para HSV-1 foram estimulados com lisado total de HSV-1, a porcentagem de células T $CD4^+$ de memória efetora ficou em torno de 0.11% no sangue, enquanto que as T $CD8^+$ foi quase indetectável (ASANUMA et al, 2000). A geração e a manutenção dos níveis desses linfócitos

e a eficiência da resposta depende de eventos da resposta inata e adaptativa. No momento da apresentação de epítopos virais pelas DCs aos linfócitos, além dos sinais co-estimuladores, a ação de citocinas, como IFN-γ, IL-2, IL-12, IL-15 e a recentemente descoberta IL-21, fazem a transição da resposta inata para adaptiva, garantindo a eficiência na produção das células de memória (SURH et al, 2005; HOFFMANN et al, 2002). Os linfócitos T $CD8^+$ de memória gerados na infecção primária estão sob constante renovação, circulam pelo organismo e acabam se infiltrando nos sítios de replicação ou latência do vírus como células efetoras de memória (STOCK et al, 2006). Entretanto, ainda não estão completamente esclarecidos os mecanismos que determinam a renovação eficiente desses linfócitos, tanto sua proliferação, quanto a função. No modelo de infecção viral crônica persistente, sabe-se que ocorre uma constante produção de células T $CD8^+$ naive (VEZYS et al, 2006). Na infecção latente, tem-se investigado a manutenção homeostática dos níveis dos linfócitos T CD8+ de memória através das citocinas, principalmente IL-7, IL-15 e IL-21 (ALVES et al, 2007). Esse fenômeno é conhecido como "bystander proliferation", e ocorre a proliferação dos linfócitos T $CD8^+$ sem a presença de antígenos (JUDGE, et al, 2002).

3- Papel da interleucina-15 e da interleucina-21 na resposta imune ao HSV-1

3.1- Interleucina 15

Interleucina 15 (IL-15) é uma citocina da família das citocinas de quatro hélices, que apresenta peso molecular de 15 kDa. Essa citocina é produzida por uma variedade de células e tecidos, incluindo placenta, músculo esquelético, rins, monócitos e macrófagos (GRABSTEIN et al, 1994). A IL-15 apresenta um receptor do tipo I (IL-15R), muito similar ao receptor da IL-2. Esse receptor é constituído de uma cadeia IL15Rα, uma cadeia IL2/15Rβ, comum com a IL-2 e uma cadeia comum às citocinas que utilizam o receptor tipo I, a cadeia gama (γ) (KAMIMURA et al, 2004). O IL15R, por sua vez, é também expresso em vários

tipos de células, linfócitos T $CD8^+$ (naive ou ativados), células NK, NKT, linfócitos Tγδ, monócitos, macrófagos, células dendríticas, neutrófilos e em células que não pertencem ao sistema imunológico, como as células endoteliais, rins, cérebro e epiteliais (KENNEDY, et al, 1996)

A resposta imune efetora específica desenvolve-se após o primeiro contato com o antígeno, sendo que, após a eliminação do patógeno, a maioria dessas células morre rapidamente por apoptose. Uma pequena porção dessas células efetoras diferencia-se em células de memória, a população que sobrevive é selecionada pela expressão do receptor de IL-7 (IL-7Rα) (KAECH et al, 2003). Nessa fase de declínio, após a retirada do patógeno, a IL-15 também regula o número de células T $CD8^+$ que sobreviverão para se tornar memória, através do aumento da expressão da proteína Bcl-2 (YAJIMA et al, 2006). Essas células de memória geradas, após a retirada do antígeno, não têm estímulo via TCR, apresentando uma razão de divisão em torno de 1 a 2 divisões a cada mês, mantendo níveis basais (MURALI-KRISHNA et al, 1999). Algumas estruturas microbianas como LPS, CpG não metilado, dupla fita de RNA são capazes de induzir a proliferação de células T $CD4^+$ e T $CD8^+$ de memória sem a ativação de TCR (GELMAN et al, 2004). Essa proliferação é indiretamente dependente de IFN α, β e IFN-γ e diretamente dependente da IL-15 (PULENDRAN et al, 1997). A IL-15 é uma citocina que está envolvida no crescimento e diferenciação de muitas células do sistema hematopoiético. Os efeitos dela ocorrem principalmente nas células de memória e são mediados pela regulação das proteínas anti-apoptóticas Bcl-2 e Bcl-X (BERARD et al, 2003). Aliás, a geração da população de células T $CD8^+$ de memória efetoras que se infiltram nas proximidades do gânglio trigêmio infectado por HSV-1 é dependente de IL-15 (SHERIDAN et al, 2006).

A IL-15 apresenta uma ação reguladora sobre outras células do sistema imune, importantes na geração da memória. Ela controla o desenvolvimento de células NK, assim

como sua homeostase. Em modelos murinos, quando é expressa em altos níveis, ocorre um aumento da percentagem, sobrevivência e citotoxicidade dessas células (YAJIMA et al, 2002). DCs geradas a partir de medula óssea em presença de GM-CSF e IL-15 são capazes de induzir uma resposta $CD8^+$ antígeno específica muito mais potente em número de células e capacidade efetora (PULENDRAN et al, 2004). Aliás, a IL-15 apresenta um mecanismo de sinalização específico, que envolve DCs e macrógafos, e que garante que as células sejam estimuladas, mesmo quando a citocina foi removia do meio. Durante um processo infeccioso, DCs ou macrófagos produzem a IL-15, que se liga no receptor IL-15Rα na superfície da célula produtora ou outra célula, o complexo é internalizado, reciclado e apresentado para uma célula T $CD8^+$ ou NK, que expressa IL-2/15Rβ e a subunidade gama (DUBOIS et al, 2002).

Outro mecanismo de controle da ação da IL-15 é em nível de transcrição, ela apresenta um sistema de expressão e secreção bem regulado. Em culturas de linfócitos observa-se que, embora eles apresentem a expressão de RNA mensageiro para IL-15, não é possível detectar a presença da citocina no sobrenadante dessas células (SATOH et al, 1998). Em estudos de transcrição e tradução gênica da IL-15, foi observado que essa citocina apresenta três isoformas que se diferenciam pelo tamanho da seqüência sinal, e que são isoformas geradas a partir de processamento alternativo (ONU et al, 1997). O processamento normal consiste na remoção dos íntrons do RNA mensageiro, quando ocorre o processamento alternativo a clivagem do RNA ocorre em um local diferente, gerando mais de uma isoforma a partir de um mesmo RNA mensageiro (WOODLEY et al, 2002). No caso da IL-15, ela apresenta oito éxons e sete íntrons, ocorrendo processamento o alternativo apenas no éxon 5. Três isoformas são produzidas, duas com a seqüência sinal de 48 aminoácidos, gerada por processamento normal e uma com uma seqüência sinal de 21 aminoácidos, gerado pelo processamento normal e alternativo. Essa isoforma gerada por *splicing* alternativo apresenta uma seqüência

sinal muito curta, além de ser constituída basicamente por aminoácidos hidrofóbicos, isso faz com que ela não seja secretada para meio extracelular, constituindo uma forma intracelular. Aliás, a forma normal, secretável, apresenta baixos índices de secreção (NISHIMURA, et al, 1998). Em modelos de camundongos transgênicos que expressam altos níveis das formas normal ou alternativa, observa-se que na forma normal, há um drástico aumento da resistência e também do número de linfócitos T $CD8^+$ de memória a infecções como *Salmonella choleraesuis, Listeria monocytogenes* e *Mycobacterium tuberculosis*, e na forma alternativa ocorre o contrário (NISHIMURA et al, 2000).

Linfócitos T $CD4^+$ de memória são parcialmente dependentes de IL-15, sendo mais dependentes de IL-7 (PURTON et al, 2007). As $CD8^+$ de memória são dependentes de IL-15 para proliferar e sobreviver (JUDGE et al, 2002). A geração de células T CD8+ de memória funcionalmente efetivas depende de sinais das células T $CD4^+$ durante a fase efetora. As $CD8^+$ de memória podem ser geradas na ausência de antígeno, em ambiente linfopênico, mas precisam da ajuda da $CD4^+$ (HAMILTON et al, 2006). Estudos com administração da IL-15 recombinante ou na forma de DNA demonstraram que a IL-15 pode substituir parcialmente a ajuda das células T $CD4^+$ em imunoterapias e imunizações (TOKA et al, 2005; KUTZLER et al, 2005). Ela tem sido intensamente aplicada na forma de terapias ou imunização com DNA para antígenos intracelulares e extracelulares, nos quais se obteve aumento na resposta citotóxica mediada por células T $CD8^+$ e também proteção ao desafio com antígenos (LI et al, 2008; TOKA et al, 2002). Uma das formas em que mais se obteve resultados foi a utilização do complexo IL-15R:IL-15Rα, que aumentou em torno de 50 vezes mais os números de células T $CD8^+$ de memória antígeno específicas (STOKLASEK et al, 2006).

3.2 – Interleucina -21

A IL-21 é uma citocina que foi recentemente descoberta, cujos efeitos no sistema imune estão começando a ser investigados. Ela foi descoberta por Parrish-Novac e colaboradores quando buscavam a identificação de um novo receptor de citocina tipo I. O receptor de IL-21 (IL-21R) apresentou propriedades proliferativas para células Baf3 quando transfectadas com uma fusão da extremidade intracelular com a extremidade extracelular de MpI (receptor de trombopoetina). Quando transfectadas com o receptor completo, as células BaF3 foram capazes de proliferar somente na presença de meio condicionado a partir de linfócitos T ativados com anti-CD3. (PARRISH-NOVAK et al, 2000). A distribuição do IL-21R foi inicialmente identificada nas células dos tecidos linfóides como baço, timo e linfonodos, sendo identificado em células B, T e NK. Entretanto, os efeitos da IL-21 já foram observados em células da linhagem não-linfóide (MAEDA, et al, 2007). A produção da IL-21 foi identificada nos linfócitos T $CD4^+$ ativados (PARRISH-NOVAK et al, 2002) e, mais recentemente, nas células NKT (COKET et al, 2007) e T_H17 (SUTO et al, 2008).

Como essa citocina é produzida pelos linfócitos, esse foi o primeiro grupo de células no qual se estudou a ação da IL-21. Ela foi capaz de estimular a proliferação em linfócitos B, mas apenas em condições de estimulação com o anticorpo anti-CD40. Nos linfócitos T ela também funciona como um co-estímulo quando as células são tratadas com anticorpo anti-CD3. Além disso, ela apresenta um efeito aditivo sobre a proliferação de linfócitos T estimulados com IL-2, IL-7 ou IL-15 (PARRISH-NOVAK et al, 2002).

A partir dessas observações iniciais, os efeitos dessa citocina em células T, B e NK têm sido intensamente investigados. As células NK são um dos principais alvos da IL-21. Após a ativação das células NK, mediada por IL-15, a IL-21 bloqueia a expansão dessas células, reduzindo a capacidade proliferativa e promovendo a indução a maturação, citotoxicidade e produção de IFN-γ, um evento ligado a transição entre a resposta inata a

adaptativa (PARRISH-NOVAK et al, 2000; KASAIAN et al, 2002). Em células NK imaturas, foi observado que a atividade proliferativa da IL-21 é dependente de doses, altas doses inibem a proliferação e baixa induzem (SPOLSKI et al, 2008). As células NK, quando tratadas com IL-21 apresentam alteração da morfologia, tornando-se maiores e granulares, perdem a expressão do marcador NK1.1, aumentam a expressão do complexo NKG2-CD94 e ativação do marcador CD154 (BRADY et al, 2004). Em estudos *in vivo*, a IL-21 é capaz de inibir o crescimento do tumor de melanoma B16 e o fibroblastoma MCA205, por um mecanismo dependente da indução de células NK (WANG et al, 2003).

Em linfócitos B, a IL-21 foi caracterizada como um fator de regulação da proliferação e produção de anticorpos. Ozaki e colaboradores produziram o primeiro *knockout* para o receptor de IL-21 (IL-21R-/-), esse animal, embora normal em número e fenótipo de timócitos, esplenócitos e células da medula óssea, apresentou altos níveis de IgE total, quando comparado com o camundongo normal, e baixos níveis de IgG1 total e específica (OZAKI et al, 2002). A IL-21 regula também a produção de anticorpos através do controle da morte e vida das células B, dependendo do estímulo que essa célula recebe. Essa regulação pode estar relacionada com o padrão de células que produz a IL-21 que estudos indicam ser a T_H2. Em uma infecção microbiana, quando as células B são ativadas através da ligação de um ligante de TLR, como LPS ou CpG, a IL-21 induz apoptose dessas células através do aumento da expressão de Bim e redução de BcL-xL. No caso de uma sinalização via BCR, a IL-21 induz a proliferação de células B. Esse mecanismo está relacionado com o controle de ativações policlonais não específicas (OZAKI et al, 2004).

A atuação da IL-21 em linfócitos T tem sido descrita como fator co-estimulador da proliferação TCR-mediada. Diferente de outras citocinas do tipo I, a IL-21 ainda não foi descrito se ela é capaz de promover a proliferação de linfócitos na ausência da ativação de TCR. A IL-21 pode ser produzida por células TCD4+ T_H1, T_H2, e T_H17, sendo que essa

última é a que apresenta maiores níveis de expressão (SUTO et al, 2008). Quanto às células T reguladoras, essa citocina tem um efeito oposto indireto, sendo que ela induz um estado de resistência da $CD4^+$ a supressão pela $CD4^+$ reguladora (PELUSO et al, 2007).

As células T $CD8^+$ são o grupo celular que recebe os maiores benefícios proliferativos da IL-21, embora ela não seja crucial para o desenvolvimento das células T $CD8^+$ (CASEY, et al, 2007). A IL-21 é capaz de agir sinergicamente com outras citocinas tipo I promovendo a proliferação de células T $CD8^+$ de memória ($CD44^{high}$) e naive ($CD44^{low}$), assim como aumentando a produção de IFN-γ por essas células. O primeiro estudo mais relevante nesse tema foi realizado por Zeng e colaboradores. Eles demonstram os efeitos sinérgicos da IL-15 e da IL-21 *in vitro* e *in vivo* utilizando um modelo tumoral, além disso, os efeitos moleculares dessa cooperação também foram descritos. O estudo demonstrou que a transcrição dos genes para granzima B, perforinas e c-jun foram aumentados na combinação (ZENG et al, 2005). Os efeitos sinérgicos também foram observados em modelo de imunização com genes de pepetídeos de HIV-1 (gene para glicoproteína Env). Nesse caso, a utilização das citocinas combinadas na forma de DNA induziu uma melhor reposta, comparado aos outros tratamentos, tanto na proliferação, quanto na proteção (BOLESTA et al, 2006). Em termos de sinalização, a IL-21 também pode atuar como um sinal adicional que previne ou resgata a expressão de CD28 em células T $CD8^+$ efetoras e de memória, que reduzem a expressão desse receptor após contato com antígeno e ação da IL-15 (ALVES et al, 2005).

Assim, a IL-15 e a Il-21 têm sido intensamente investigadas para o desenvolvimento de sistemas terapêuticos e vacinas, baseadas na otimização da função e proliferação de células T $CD8^+$. Os mecanismos de interação ainda não estão bem detalhados, mas os dados atuais são encorajadores para o desenvolvimento de pesquisas com esse tema.

4 -Objetivos

4.1 - Objetivo geral:

Avaliar o potencial da IL-21 e da IL-15 aplicadas na forma de DNA como adjuvantes na otimização da resposta $CD8^+$ *in vitro* e *in vivo* com um modelo de infecção viral com o HSV-1.

4.2 - Objetivos específicos:

1) Investigar se as citocinas IL-15 e IL-21 produzidas de forma recombinante influenciam a freqüência de células T $CD8^+$ in vitro.
2) Investigar se os plasmídeos construídos para expressar as citocinas IL-15 e IL-21 melhoram a resposta $CD8^+$ efetora primária na infecção com HSV-1.
3) Determinar as quantidades mínimas de células SSIEFARL transgênicas necessárias para transferência e expansão, a fim de gerar células de memórias para os estudos com os plasmídeos das citocinas.
4) Determinar se pIL-15 e pIL-21 são capazes de induzir a proliferação de células T $CD8^+$ de memória in vivo, utilizando o sistema de transferência de células transgênicas.

CAPÍTULO II

IL-21 administration as plasmid DNA increases the numbers of HSV-1 specific effector and memory $CD8^+$ T cell and IFN-γ production.

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Full title: IL-21 administration as plasmid DNA increases the numbers of HSV-1 specific effector and memory $CD8^+$ T cell and IFN-γ production

Running title: IL-21 expands memory $CD8^+$ T cells

Luiz Rodrigues*, Subhadra Nandakumar**, Cristina Bonorino *, Barry T. Rouse*** and Uday Kumaraguru**

*Faculdade de Biociências e Instituto de Pesquisas Biomédicas
Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil
** Department of Microbiology, Quillen College of Medicine, ETSU, Johnson City, TN-37614
*** Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN-37996

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Abstract

Herpes Simplex Virus-1 (HSV-1) causes a latent infection in the neurons. The recurrence of lesions and transmission are dependent on the number and function of viral specific $CD8^+$ T cells, especially the memory T cells. The generation, turnover and set point of this cell population is maintained by different factors like exposure to antigen, cytokines and co-stimulatory molecules. However, the contribution of these factors in the generation and maintenance of the memory $CD8^+$ T cell population is still controversial, since it is not clear if homeostatic proliferation driven by cytokines can overcome T cell receptor (TCR) signaling. Interleukin 15 (IL-15) and interleukin 21 (IL-21) are cytokines implicated in homeostatic control of $CD8^+$ T cell pool. We constructed expression plasmids coding for IL-15 (pIL-15) and IL-21 (pIL-21) and used them to expand HSV-1 specific $CD8^+$ T cells (SSIEFARL) in an adoptive transfer model. Our results showed that transfer of low cell concentrations is required to obtain high numbers of new effector cells following viral infection. *In vitro*, IL-21 increased the frequency of $CD8^+$ T cells in the absence of antigen, although the magnitude of this response was dependent on TCR signaling. Both pIL-15 and pIL-21 boosted the numbers of antigen specific $CD8^+$ IFNγ producing cells in the primary response. In the memory phase, numbers of $CD8^+CD44^{high}$ as well as $CD8^+$IFNγ producing cells were increased when pIL-15 and pIL-21 were used alone or in combination, compared to vector treatment only, and association of antigen slight increased the proliferation response. Our data suggest that genetic treatment with pIL-15 and pIL-21 can contribute to boost HSV-1 specific $CD8^+$ effectors cells in primary responses. During the memory phase of HSV-1 infection, pIL-21 or pIL-15 can increase the frequency of antigen specific $CD8^+CD44^{high}$ cells. The inclusion of antigen did not compensate the genetic cytokine treatment; however the combination of pIL-21, pIL-15 and antigen was more efficient.

1. Introduction

One of the main goals of vaccine research is to maximize the generation, maintenance and function of memory $CD8^+$ T cells, determinant for the immune responses against viruses and intracellular pathogens [1]. Memory $CD8^+$ T cells express high levels of CD44 and can be divided in two populations based on expression of homing molecules: central memory cells ($CD44^{high}$ $CD62^{high}$), located in lymphoid organs such as lymph nodes and spleen, and effector memory cells ($CD44^{high}$ $CD62^{low}$), that circulate between lymphoid organs, tissues and blood [2] [3] . These two populations of memory $CD8^+$ T cells are generated after the contraction phase of $CD8^+$ T cells, this population is maintained and constantly renewed by basal proliferation and is known as the memory set point [4]. Different infectious agents such as viruses or bacteria can generate different set points of specific memory $CD8^+$ T cells, that can be expanded and perform effector functions faster and more efficiently than recently recruited effector $CD8^+$ T cells [5]. In the case of Herpes Simplex Virus-1 (HSV-1), the set point of memory $CD8^+$ T cells is less than 1%, and that can be disadvantageous to the host because this virus produces a latent infection, the control of reactivation being fully dependent from memory $CD8^+$ T cells [6] [7]. This set point of memory $CD8^+$ T cells is maintained by different factors such as low doses of antigen or cytokines [8-11]. However it is still unclear which factors are determinant in this mechanism, more so in the context of a latent infection such as HSV-1.

Interleukin-15, a four-helix bundle cytokine, has a pivotal role in the control of life and death of lymphocytes, especially memory $CD8^+$ T lymphocytes [12-14]. It is well documented that naive $CD8^+$ T cells do not express high amounts of IL-15Rα, however these cells can respond to IL-15 signaling. The response is mostly mediated

through the recycling of IL-15/IL-15Rα complex by monocytes and dendritic cells and transpresentation of it to $CD8^+$ T cells that do express IL-2/15Rβ and the γ chain[15].

There are also other cytokines that act like adjuvant, contributing to potentiate the formation and maintenance of an effective $CD8^+$ T cell memory pool [16-19]. Interleukin-21, a new member of a family of cytokines that uses receptors containing the common γ-chain is one of adjuvants that participate in the memory $CD8^+$ T cell pool formation and maintenance [20-22]. This cytokine is produced by activated $CD4^+$ T cells and NKT cells, indicating that it is involved in adaptive immunity [23-25]. The adjuvant effects of IL-21 on viral immunity were first observed by Cui and collaborators, who showed that immunization with a plasmid coding for the HSV-1 CD8 epitope (gB) together with IL-21 gene protected mice from lethal HSV-1 challenge [26]. The protective effects were related to the capacity of antigen specific $CD8^+$ T cell to produce IFN-γ. IL-21 is involved in the differentiation of naive CD8+ T cells but require IL-2 to support the development of effector functions [27]. Additionally, *in vitro* and *in vivo* assays suggest that IL-21 has a role in proliferation and maturation of natural killer (NK) cell populations, proliferation, survival and antibody production of mature B-cells, as well as in the proliferation of T cells co-stimulated with anti-CD3 [20, 28, 29]. When DCs are treated with IL-21 they maintain their immature phenotype after antigen uptake and LPS stimulation, and the expression of MHC class II and CCR7 is reduced [30, 31]. Taken together these results suggest that IL-21 modulates the transition between innate to adaptive immunity.

It remains to be determined how IL-21 can help and what are the kinetics of its function in order to design a therapeutic program. The combination of IL-21 with IL-15, IL-7 or IL-18 can increase the numbers and function of effector and memory

$CD8^+$ T cells [32]. There are several studies showing an evident synergism between IL-21 and IL-15 [22, 32, 47, 56]. In this study we constructed a plasmid encoding murine IL-21 (pIL-21) and murine IL-15 (pIL-15) to investigate the effects of these cytokines on the proliferation and function of $CD8^+$ T cells in the absence or presence of antigen when used as a DNA treatment. We found that pIL-21 can increase $CD8^+$ T cell numbers *in vitro* in non-activated as well as activated cells. In vivo, DNA treatment with pIL-21 and pIL-15 contributes to improve the generation of the first population of $CD8^+$ IFN-γ^+ cells in an HSV infection model. During memory phase, treatment with pIL-21 or pIL-15 alone was sufficient to expand memory HSV-1 $CD8^+$ TCR specific cells. The combination of the two cytokines DNA or antigen DNA affected proliferation and IFN-γ production of antigen specific memory $CD8^+$ T cell similarly. However, all the cytokine plasmid treatment were better than the vector alone. Our results suggest that administration of IL-21 and IL-15 as DNA may bypass the need for antigen supply for the expansion of memory $CD8^+$ T cells in a viral latent infection.

2. Materials and Methods

2.1 Mice

Female C57BL/6 mice were purchased from Charles River. Mice were maintained according to the Guide for the Care and Use of laboratory Animals (National Academy Press, Washington DC, 1996). Animals were kept in specific pathogen-free conditions in the Laboratory Animal Facility, University of Tennessee, and Division of Animal Resources, College of Medicine, East Tennessee State University which is

fully accredited by the American Association for Accreditation of Laboratory Animal Care.

2.2 Cell line and viruses

Vero African green monkey kidney cells (ATCC cat n CCL81)] and H293 (human kidney embryonic) cell lines were cultured in Dullbecco's minimal essential medium (DMEM;Gibco-BRL) supplemented with 10% fetal bovine serum (FBS). CTLL-2 cells (ATCC cat n TIB 214) were grown and maintained in RPMI complete medium containing 5% supernatant from Con-A-stimulated rat spleen cells (BD Biosciences) supplemented with IL-2, which is required for cell growth. HSV-1- KOS were cultured and titrated on a Vero cell monolayer, and the supernatant stored in aliquots at -80 ^{0}C until use.

2.3 FACS and ELISA

Cy-chrome-conjugated anti-mouse CD8, PE-conjugated anti-mouse CD4, FITC-conjugated anti-mouse CD3, FITC-conjugated anti-mouse IFN-γ, APC-conjugated anti-mouse CD90.1, FITC-conjugated anti-mouse CD8, Cy-chrome-conjugated anti mouse IFN-γ, PE-conjugated anti-mouse TNF-α, PE-conjugates anti-mouse CD8, FITC-conjugated. Cy-chrome-conjugated anti-mouse CD44 were purchased from BD Biosciences. PE- MHC Class I (*H*-2^{d}) tetramers used to measure SSIEFARL specific T cell were provided by NAIAD MHC Tetramer Core Facility (Atlanta, GA). Cytokines were measured by ELISA using polyclonal anti-IL-15, polyclonal anti-IL-21, biotin-conjugated IL-15, biotin conjugated IL-21, recombinant protein IL-15 and recombinant protein IL-21, all purchased from R&D and used according to the manufacturer's recommendation. For intracellular staining for IFN-γ, spleen cells

were isolated from immunized or non-immunized mice at appropriate times. Cells ($1x10^6$/well) were plated in V-bottomed microwell plates in RPMI 10% FBS supplemented with 40 U IL-2 per well and stimulated with SSIEFARL peptide (1μg/well) in presence of Golgi Plug transport inhibitor. The splenocytes were incubated for 5 h at 37°C, 5% CO_2 and stained for CD8 and intracellular IFN-γ after permiabilizing the cells using permfix. Flow cytometry was performed in a Becton Dickson FACScan, and the data were analyzed with FloJo software (Treestar).

2.3 Plasmids constructions

RNA for murine IL-15 (mIL-15) was obtained from macrophages stimulated for 6 hours with lipopolysaccharide (LPS) to induce gene transcription [33]. mRNA was extracted using a Qiagen RNeasy kit and cDNA was synthesized. The IL-15 forward primer (5' cattgaattccttacctgggcattaagtaatgaaaattt 3') and the reverse primer (5' aggctctagagcagtcaggacatgttgatgaacatttg 3') were used to amplify the specific cDNA. Restriction sites for *Eco*RI and *Xba* I were inserted on the forward and reverse primers, respectively. PCR conditions for all reactions were one cycle at 94ºC for 2 minutes; 30 cycles at 94ºC for 1 minute, 56º for 45 seconds, and 68º for 1 minute, followed by a final extension step at 68º for 5 minutes. All reactions were performed using high fidelity *Pfx* polymerase (Invitrogen). The final product was migrated in 1.5% agarose gels for further purification. The murine interleukin-21 (IL-21) expression plasmid (pORF-mIL-21) was purchased from Invivogen and the gene excised using *Nco* I and *Nhe* I. For plasmid DNA preparation DNA encoding IL-15 and IL-21 was isolated from agarose gels using Gene clean kits (Q-Biogene). The DNA was digested with restriction enzymes (Fisher) and cloned into the pVIVO-2

vector under control of ferritin promoter (Invivogen) or pCR3.1 vector under control of cytomegalovirus promoter (Invitrogen). The plasmid encoding the whole sequence of HSV-1 glycoprotein B (pgB) was a gift from Dr. Ozma, The University of Japan. All positive clones obtained after ligation and transformation in *E.coli* F5-alfa (Fisher) cells were sequenced and clones that presented 100% of identity with mIL-21 or mIL-15 sequences available in GeneBank were tested for *in vitro* expression. All plasmid DNA constructions were purified using Plasmid Endofree Mega prep (Qiagen). The isolated plasmids were precipitated in 3M Sodium acetate and absolute ethanol, and ressuspended in 1X PBS.

2.4 Immunization and Infection

DNA was injected in PBS 0.25% bupivacaine (SIGMA) in a final volume of 100 μL. Animals were anesthetized intraperitoneally with 200 μL of avertin and immunized in the tibial muscle with 75 μg of pgB alone or in combination with pIL-15 and/or pIL-21. The empty vector was used as a control. Mice were infected intraperitoneally with 5 $x10^6$ PFU of HSV-KOS or in the tibial muscle with 5 x 10^4 PFU.

***2.5 In vitro* Expression**

Expression levels of the plasmid constructions were tested after transient transfection of H293 cells. Cells were plated in six-well tissue culture plates at a density of 2 $x10^5$ cells/well in complete DMEM plus 10% FBS and allowed to adhere overnight. The next day, medium was replaced by serum free-DMEM and the cells were transfected with each plasmid construction using lipofectamine 2000

(Invitrogen). The plate was incubated for 6 hours at 37ºC with 5% CO_2 and the medium was replaced by DMEM 10% FBS. After 72 hours, supernatants were harvested and analyzed for the presence of murine IL-15 by ELISA using capture and detection anti-mouse IL-15 or anti-mouse IL-21 monoclonal antibodies (R&D systens). Expression of the gB plasmid was analyzed by immunoblotting with monoclonal antibody anti-gB (Virusys).

2.6 In vivo Expression

Mice were injected intramuscularly with each plasmid construction previously described. Three days after injection, the tibial muscle was dissected, minced and the cells cultured in DMEM 1X 10% FBS for three more days at 37^0C, 5% CO_2. The supernatant was collected and tested by ELISA using capture and detection anti-mouse IL-21 or IL-15 antibodies.

2.7 In vitro activity cytokines

Seventy two hours after transfection the supernatants of H293 cells transfected with pIL-21, pIL-15 or control vector were removed and concentrated in centricon filters - 3 (Millipore). Protein concentration was determined by ELISA. These concentrated supernatants of IL-21, IL-15 or control vector transfected cells were used to supplement cell culture medium. Forty ng/μL of concentrated supernatant of IL-21 were added to RPMI (10% FBS supplemented with antibiotics, 50 μM β-mercaptoethanol and 50 U/mL of recombinant human IL-2 [Hemogen]. The same volume of concentrated vector-transfected cell supernatant was used. Media with recombinant IL-21 (R&D Systens) (40 ng/mL) or PBS were used as control media.

Single cell suspensions of spleen were prepared by gently pressing the tissues through fine nylon screen. Erythrocytes were depleted with Red Cell Lysis Buffer (SIGMA). Cells were plated in 48 well plates at 1 x 10^6 cells/well and cultured using control medium or medium with IL-21. After 6 days of incubation at 37^o C with 5% CO_2, cells were harvested and the T cells were analyzed by flow cytometry. To test the effect of IL-21 on activated splenocytes the experiment was repeated and cells co-incubated with anti-CD3 0.25 μg/mL.

The IL-2 dependent murine T cell line CTLL-2 was used to assess biological activity of mIL-15 encoded by the plasmid constructions in vitro. CTLL-2 cells obtained from American Type Culture Collection were grown and maintained in RPMI 1640 (Sigma) added of 10% Fetal bovine serum and 50U/mL of IL-2, which is required for cell growth. At the time of assay, cells were centrifuged and washed four times with RPMI 1640 to remove residual IL-2. Cells were plated at a concentration of 5 X10^4 cells/well in 96 well, flat bottom polystyrene plates. Commercial murine IL-15 (eBioscience) as well as the concentrated supernatants from transfected cells was added to each well. The cells were incubated in incubator with 5% CO_2 at 37^{o}C for 48h. For analysis of proliferation, (methyl-3H) thymidine was added to triplicate wells and incubated for 8 hours.

To determine if the effects observed with IL-21 in T cells after treatment of splenocytes with IL-21 supernatants was a specific effect, an antibody neutralization method was used. The media described above were treated for one hour with different concentrations of anti-murine IL-21 antibody (15, 25 or 50 μg/mL) to neutralize the IL-21 protein and block it to binding to its receptor on cells surface. Six days after culture $CD3^+CD8^+$ cell numbers were analyzed by flow cytometry.

2.8 Adoptive Transfer

Splenocytes from HSV-1 gB TCR specific transgenic C57BL/6, CD 90.2 (Thy 1.2)$^{+}$ (gBT cells) mice were isolated, reticulocytes lysed with RBC buffer (Sygma) and 1 X 10^{8} cells stained with 5 μM of CFSE in 4 ml of PBS, 37º C, 5% CO_2 for 7 minutes. The reaction was stopped with 4 mL of cold FBS and kept on ice for 2 minutes. Cells were washed three times with 4 mL of PBS 2% FBS. CFSE stained Splenocytes were injected intravenously (i.v.) by the tail vein in C57BL/6 CD90.1^{+} (Thy 1.1)$^{+}$ mice.

2.9 Data Analysis

All the experiments were performed in at least three independent assays, unless specified. Data were tested for normality of distribution by a Kolmogorov-Smirnov test, and depending on the result, differences were analyzed either by parametric tests (Student's t test and ANOVA, with Tukey post tests) or non-parametric tests (Mann-Whitney and Kruskall-Wallis, with Dunn post tests). A value of $p<0,05$ was chosen as a level of significance. Analysis was conducted using GraphPad Prism 4.0 Software.

3.Results

3.1. IL-21 and IL-15 can be efficiently expressed in mammalian cells under a ferritin promoter

We first set out to determine if our constructs were efficiently expressed in mammalian cells, both *in vitro* and *in vivo*. We initially tested the efficiency of expression of IL-21 and IL-15 under the control of two different mammalian expression promoters, the well-studied cytomegalovirus (CMV) promoter cloned into pCR 3.1 plasmid, and the less used ferritin promoter, cloned into the pVIVO-2 plasmid. To test *in vitro* expression, we transfected H293 cells with either pIL-21 or pIL-15, or control vector only, as described in the methods section. After 72 h of culture, supernatant was collected and tested for cytokine expression by ELISA. The ferritin promoter used in these constructs led to higher yields of cytokine production compared to what was obtained using the CMV promoter *in vitro* (Figure 1A and 1B). To analyze *in vivo* expression, mice were immunized intramuscularly with pIL-21 or pIL-15 plus bupivacaine. After three days, muscle was dissected and cultured for another three days, to measure cytokine expression in the supernatant [34]. While we could easily observe an increase in IL-21 expression in vivo (Figure1C), we could not verify a clear increase in IL-15 expression compared to injection of vector alone for (results not shown). This could be due to the high IL-15 production by tissue macrophages and dendritic cells in response to DNA injection [35].

3.2.Supernatants of pIL-21 and pIL-15 transfected cells have an effect on $CD8^+$ T cell numbers *in vitro*

After we determined that the cytokines encoded by our constructs were efficiently expressed in vitro and in vivo, we investigated if the cytokines produced by the cells transfected with our constructs could influence $CD8^+$T cell numbers in vitro. To analyze the effect of IL-21, the supernatant of H293 cells transfected with pIL-21 (pIL-21H293 cells) was concentrated, and used to supplement an IL-2 conditioned RPMI media. Splenocytes of naive C57Bl/6 mice were cultured in this media for 6 days at 37^{o}C and 5% CO_2. As positive controls, some cells were cultured with RPMI supplemented with IL-2 or commercial IL-21. On the seventh day the cells were harvested, counted and analyzed by flow cytometry. The results as shown in Figure 2A-D, indicate that the number of $CD3^+CD8^+$ T cells increased in cultures with IL-21, (both commercial and produced by pIL-21H293 cells) without the engagement of TCR. In fact, when the cells were treated by 30 minutes with anti-CD3 and cultured in the presence of IL-21, the effects on $CD3^+CD8^+$ T cell expansion were more intense, especially when pIL-21H293 was used in the culture. Figure 2E shows the absolute numbers of the cultured cells, demonstrating a increasing of $CD3^+CD8^+$ T cells in the IL-21 treated cells. To investigate, if the in vitro proliferation of $CD8^+$ T cells cultured in media supplemented with pIL-21H293 is a phenomenon specifically related to IL-21, pIL-21H293 supernatants were treated for one hour with three different concentrations of murine anti-IL-21 antibody (15 µg/mL, 25 µg/mL or 50 µg/mL) and used to supplement RPMI. Six days after culture, $CD3^+CD8^+$ T cells numbers were analyzed. As seen in Figure 2F, the numbers of $CD3^+CD8^+$ T cells in the wells treated with anti-IL-21 decreased compared to wells without neutralizing treatment. The $CD3^+CD8^+$ T cells numbers decreased proportionally to the amount of anti-IL-21

added. Taken together, these results indicate a direct involvement of IL-21 cytokine in the expansion of $CD3^+CD8^+$ T cells *in vitro*.

Using this same assay we observed that IL-15 did not have an effect over the proliferation of naive cells (results not shown), probably because naive cells don't express high levels of IL-15Rα and its activity on this cells is mainly mediated by transpresentation of this receptor subunit by macrophages or dendritic cells to IL-2/IL-15RB of $CD8^+$T cells [15, 36]. Consequently, we used a CTLL-2 cell proliferation assay, because these cells are highly dependent on IL-15 to proliferate. CTLL-2 cells were cultured in RPMI supplemented with IL-2 until cells were approximately 80% confluent, washed several times to thoroughly remove residual IL-2 and cultured with RPMI supplemented with supernatant from pIL-15H293 cells for 48h and T^{3H} - thymidine was added for another 8h. CTLL-2 proliferated when cultured in presence of pIL15H293 supernatant proportionally to the concentrations of IL-15 present in media (Figure 3). This result showed that the IL-15 encoded by our constructions can efficiently promote the proliferation a cytotoxic T cell that constitutive express IL-2/15Rβ and IL-15Rα.

3.3 – pIL-21 and pIL-15 can potentiate the numbers of antigen specific IFN-γ producing $CD8^+$ T cells in the primary response *in vivo*

Since the IL-21 and IL-15 encoded by the plasmid constructions respectively increased the frequency and promoted proliferation of $CD8^+$ T cells in vitro, we decided to investigate their role in the priming phase of adaptive immunity *in vivo*, using an HSV infection model. Mice were infected intraperitoneally on day zero with 5 $x10^4$ *PFU* of HSV-KOS and two days after infection they received an intramuscular injection with pIL-21 or control vector. Each was treated with 75 μg of pIL-21 or

control vector diluted in PBS 0,25% bupivacaine on the right leg. Twelve days after infection, mice were sacrificed and spleens were harvested. The organs were minced, spleen RBC lysed and counted, and single cell suspensions were washed with RPMI, counted and cultivated for 5 h with gB peptide. The cells were then stained for CD8 and IFN-γ, as described in materials and methods.

As seen in Figures 4C, in spleens from mice infected and treated with pIL-21 the percentage of cells $CD8^+$ IFN-γ^+ was almost two times higher than cells from mice infected and treated with vector alone (figure 4B). Also, when absolute numbers of CD8+IFN-γ cells were analyzed on total splenocytes from infected mice and treated with vector or pIL-21, the response didn't differ between uninfected and vector treated, but was different between pIL-21 treated and uninfected ($p<0.01$) (Figure 4E). This result agreed with what was observed in the *in vitro* experiments, supporting that IL-21 can influence the frequency of $CD8^+$ T cells *in vivo* and suggesting that it can potentiate the generation of cytotoxic T cell effectors in the beginning of immune response.

We repeated the same immunization experiments with pIL-15, and as seen in Figure 4D the percentage of $CD8^+$ IFN-γ^+ T cells on day 12 after immunization was higher in mice that received pIL-15 treatment compared with mice that received vector only. Results similar to pIL-21 treatment was observed when absolute number of $CD8^+$IFN-γ^+ T cells was analyzed, mice treated with vector was not different from uninfected mice, but a difference was observed between pIL-15 vs unincfected ($p<0.05$) (Figure 4F). Taken together these data suggest that DNA immunization with pIL-15 and pIL-21 treatment during the beginning of primary immune response can accelerate the effector phase.

3.4 Determination of the optimal number of SSIEFARL CD8+ cells to be used in an adoptive transfer system

To study the effects of pIL-21 and pIL-15 in memory $CD8^+$ T cell generation and maintenance *in vivo,* we used $CD8^+$T cells from HSV-SSIEFARL transgenic mice, since these cells respond quickly and efficiently after HSV-1 infection and also the numbers of cells produced are high and easily detected. SSIEFARL is a HSV-1 (gB glycoprotein) CD8 epitope. The population of memory T cell as well as naive T cell is highly influenced by the clonal abundance of initial precursors, since the proliferation of these cells is benefited from a low clonal abundance [37]. Hence, as a first step we sought to determine the optimal amounts of total splenocytes from $CD90.2^+$ SSIEFARL transgenic mice to be transferred, by analyzing proliferation rates after parking them into recipient mice. In order to do this, we labeled total splenocytes of $CD90.2^+$ SSIEFARL mice with CFSE, adoptively transferred intravenously different amounts of these cells into C57BL/6 $CD90.1^+$ recipient mice and infected the recipient mice intraperitoneally with 5 x 10^6 PFU of HSV-KOS. Before transfer, the numbers of SSIEFARL $CD8^+$ T cells present in total splenocytes were determined by FACS using SSIEFARL tetramer-PE and anti-CD8-FITC staining. Groups of C57BL/6 $CD90.1^+$ received 2 x10^3, 2 x10^4, 2x10^5 or 1 x 10^6 CFSE labeled splenocytes. Each dose of total splenocytes transferred represented 2.88 x 10^2, 2.88 x10^3, 2.88 x10^4 and 1.44 x10^5 SSIEFARL CD8+ T cells, respectively. A group of mice was sacrificed seven days after infection. Splenocytes were stained with APC labeled anti-CD90.2, PE- labeled SSIEFARL tetramer and Cy-Chrome labeled anti-CD8, for FACS analysis.

The data in Figure 5 shows the fold increase in SSIEFARL specific $CD8^+$ T cell population recovered seven days after transfer and viral infection. When we adoptively transferred $1,44X10^5$ SSIEFARL $CD8^+$ T cells ($1 X 10^6$ total splenocytes) the fold expansion of these cells was around 7.9 times. Interestingly, transfer of very low numbers of SSIEFARL $CD8^+$ T cells ($2,88 x 10^2$) yielded a 902 fold increase in the donor transgenic population, a highly significant expansion (R2 0.9469, $p<0.0001$). Taken together these results indicate that a low transfer of SSIEFARL cells is more efficient for generation of new antigen specific responder $CD8^+$ T cell.

3.5 pIL-21 and pIL-15 increases the memory $CD8^+$T cell population *in vivo*

Once we arrived at the number of splenocytes to be transferred to produce a new progeny of $CD8^+$ T cells and the formation of stable memory T cells, we adoptively transferred $2 x 10^4$ donor SSIEFARL $CD90.2^+$ total splenocytes into C57BL/6 $CD90.1^+$ recipient mice. The recipient mice were infected with HSV-KOS and kept for 60 days to reach memory phase. Upon memory phase each group of mice were injected into tibial muscle with 75 μg of each cytokine plasmid construction alone, combined or associated with pgB (plasmid encoding HSV-glycoprotein B); vector only was used as a control. Ten days after DNA treatment the mice were sacrificed, splenocytes were isolated and the cells analyzed by FACS. The experimental design is represented on figure 6A. Figure 6B shows histogram plots a $CD44^+$ population gated from $CD90.2^+$ $CD8^+$ $SSIEFARL^+$ cells. The Mean Fluorescence Intensity (MFI) is represented for each group. Mice treated with pIL-21 and pIL-15 presented higher expression of CD44 on transgenic $CD8^+$ T cells compared with groups treated with vector only. This result showed that $CD8^+CD44^{high}$ memory population was more frequently on pIL-21 as well as pIL-15 alone but the

combination didn't improve the levels reached by the exogenous treatment with each cytokine. Also when we associated antigen (pgB) to the immunization, CD44 MFI didn't change significantly in $CD8^+$ cells compared with the cytokines alone.

When we calculated the absolute numbers of a CD8+ T cells inside a population of CD90.2+ cells isolated from spleen of each group plasmid treatment, a difference between pIL-21 and pIL-15 treated groups compared with vector alone could be seen (Figure 6C). We didn't see difference among cytokines plasmids treated groups alone. Antigen provided by pgB plasmid didn't improve significantly the number of these cells when associated with pIL-21 or pIL-15. However, the combination pIL-21, pIL-15 and antigen were slightly better. Plasmid coding IL-21 or IL-15 alone was sufficient to increase the numbers of total $CD8^+$ T cells within the $CD90.2^+$ population compared with vector treatment, and when we combined pIL-21 and pIL-15 with pgB the effects on $CD8^+$ T cells were more. Since SSIEFARL peptide is an HSV-1 $CD8^+$ T cell exclusive epitope, that can be determinated by MHC I tetramer (tet) and $CD44^{high}$ is a memory marker, we also measured the population of $CD44^{high}Tet^+$ cells within $CD90.2^+$ population. Again, the magnitude of cells recovery from mice treated with pIL-21 and pIL-15 alone or in combination was higher then what was recovered from mice that received vector only (Figure 6D). The groups of pIL-15+gB and pIL-21+pIL-15+pgB demonstrated higher percentage (data not shown) as well as numbers of $CD44^{high}Tet^+$ cells suggesting some additional contribution of IL-15 or antigen to this population of cells. The results from these experiments show that the numbers of antigen specific memory $CD8^+$T cells was positively influenced by presence of pIL-21 and pIL-15 during memory phase. Cytokine plasmids treatment increases the frequency of antigen specific $CD90.2^+$ cells with memory phenotype.

Once the numbers of antigen specific $CD8^+$ T cells recovered during memory phase were increased by plasmid cytokine treatment we investigated the functionality of these cells analyzing the percentage of $CD8^+$ IFN-γ producing cells. Splenocytes from each group of mice were intracellular stained with anti-IFN-γ and surface stained with anti-CD8 (Figure 6E). Mice treated with pIL-21 alone have around 2 times more CD8+IFN-γ^+ cells than mice treated with vector only. The same effect could be seen on cells from mice treated with pIL-15, although with more magnitude. The numbers of CD8+ IFN-γ^+ cells produced by mice treated with the combination of pIL-21 and pIL-15 were not different from what was generated by treatment of each alone. Antigen (pgB) association improved the $CD8^+IFN\gamma^+$ T cell response, especially with the combination of the pIL-15 + pIL-21 and pgB.

Taken together these results show that pIL-21 and pIL-15 injection, alone or in combination during memory phase results in an additive effect on memory $CD8^+$ T cells numbers, even in absence of antigen. HSV-specific $CD8^+$T cells population were increased in numbers upon plasmid cytokine delivery. Therefore, more IFN-γ producing cells are seen, maybe due to the increase frequency of HSV-specific memory T cells.

4 - Discussion

In this study we attempted to determine if murine IL-21 alone or in combination with murine IL-15 could expand the HSV-1 antigen specific memory $CD8^+$ T cells by a homeostatic mechanism. To test this hypothesis we used genetic therapy with both cytokines under control of ferritin promoter, which we established to be more efficient than the CMV promoter *in vitro*.

Our main objective was to investigate the roles of pIL-21 and pIL-15 on $CD8^+$ T cells. We tested if the cytokines present in the supernatant of cells transfected with each plasmid could expand lymphocytes isolated from naive mice. As shown in Figure 2, the supernatant from cells transfected with pIL-21 increased the frequency of $CD3^+CD8^+$ T cells two-fold, compared with IL-2 alone. Indeed, IL-21 can efficiently expand $CD8^+$ T cells, but this cytokine needs help from other γ chain-receptor using cytokines (IL-2, IL-7, IL-15 or IL-18) to promote this expansion in the absence of TCR stimulation [22]. In contrast, when we associated anti-CD3, the increase in frequency of $CD3^+CD8^+$ cells was highly significant (Figure 2E). These results agree with the first studies with IL-21 reported by Parrish-Novak et al., which showed that the IL-21 receptor (IL-21R) is expressed by $CD8^+$T cells, and this cytokine acts in concert with IL-2 to promote T cell proliferation [20]. Recent studies have shown that the IL-21R is upregulated on T lymphocytes in response to either TCR stimulation or IL-21 [38, 39]. Also IL-21 augments the frequency of antigen-specific $CD8^+$ T cells following primary *in vitro* stimulation around twenty-fold, compared with no IL-21 addition [40]. Is also possible that IL-21 acted in synergy with IL-2 produced by activated $CD4^+$ T cells, and that may have affected the $CD8^+$ T cells expansion.

We also tested the supernatant of cells transfected with pIL-15 on splenocytes in vitro, however no improvement in $CD3^+CD8^+$ T cell frequency was observed. This agreed with previous reports showing that naive murine $CD8^+$ T cells do not respond to human or mouse IL-15 stimulation in vitro, unless antigen is present [41]. In contrast, human $CD8^+$ T cells proliferate under stimulation with IL-15 *in vitro*, without the need for antigen [42, 43]. Also, the main mechanism to $CD8^+$ T cells respond to IL-15 is through trans-presentation by DCs. Maybe DCs died or didn't present this function under cultured conditions.

We tested if these findings would be reproducible *in vivo*, during the priming of an immune response to HSV-1 infection. An increase in effector $CD8^+$ IFN-γ producing cells was observed with cytokine plasmid treatment. Interleukin-21 lead to an increase in antigen-specific effector $CD8^+$ T cells during the expansion phase of viral specific $CD8^+$ T cells. The improved antigen specific effector $CD8^+$ T cell response to IL-21 treatment has been seen in other studies also. It has been observed that IL-21R signaling activates PI3K pathway and induces Bcl2 expression, promoting $CD8^+$ T cell survival (Ostiguy, 2007). Also, the benefits of IL-21 treatment on antigen-specific $CD8^+$ CTL frequencies during the effector phase were observed in tumor models as well as in cytomegalovirus vaccination studies [44, 45]. Our results are in agreement with previous reports that showed that injection of an IL-21 coding plasmid alone increases the percentage and absolute numbers of $CD3^+$ and $CD8^+$ cells [46], and IL-21 administration during the early phase of the immune response confers benefits to antigen specific $CD8^+$ T cell response [47].

We found similar results when mice were infected and treated with pIL-15. Although the main targets for IL-15 are memory $CD8^+$ T cells, this cytokine can also regulate $CD8^+$ T cells during priming phase. IL-15 is an essential factor for rescue of $CD8^+$ T cell from apoptosis during the contraction phase, after infection with an intracellular pathogen [48]. Interleukin-15 is also one of the factors involved in survival of $CD8^+$ T cells and its effects are mediated by induction of expression of Bcl-2 anti-apoptotic members [13]. Indeed, IL-15 was already described as an adjuvant to CTL responses [49-52]. There is possibility that effectors $CD8^+$ T cells had been saved from apoptosis by the IL-15 produced after plasmid administration.

We next tested the effect of genetic delivery of pIL-15 and pIL-21 during the memory phase of the response, using an adoptive transfer system of CD90.2/gBT

cells. Because the *in vivo* clonal expansion of effector priming population after antigen stimulation influences the magnitude of the memory cell population, we had to determine the optimal number of total splenocytes from SSIEFARL CD90.2$^+$ cells to be transferred into regular C57BL/6 CD90.1 mice. As seen on Figure 5, when a high amount of SSIEFARL CD8+ T cells were transferred (1.44 x 10^5), a 10 fold expansion was observed; contrastingly, when a low amount was transferred (2.88x10^2), a 900 fold increase was observed. This result agreed with the finding that a low cell frequency is important for the formation of a stable effector memory CD8$^+$ T cell pool [53], and stressed the importance of clonal competition among antigen specific precursors for survival signals provided by TCR recognition, as reported by Hataye et al [37]. We consequently performed adoptive transfers with low numbers of SSIEFARL CD8$^+$ T cells (2.88 x10^3), because it favored the survival and activation of CD8$^+$ T cells.

The administration of plasmids coding for IL-15 or IL-21 alone or in combination during memory phase of HSV-1 infection lead to an increase of expression of CD44 on CD90.2$^+$CD8$^+$ cells as shown in Figure 6B, neither of these treatments being significantly superior to each other, only when compared with the vector. Also, when pgB was associated with the cytokine DNA treatment no significant difference in magnitude of CD44 was observed compared to the cytokine treatment alone. Numbers of CD8$^+$ T cells that expressed T memory and HSV-specific markers (CD44 and SSIEFARL tetramer) in a monoclonal population was augmented by pIL-21 or pIL-15 alone or in combination. However when pIL-15 and pgB was used, some groups showed some additional improving to the number of cells recovered as seen on Figures 6 D. It was expected since IL-15 is well studied as a memory proliferation factor, especially to CD8 and these cells do express the IL-

15 receptor complex [54, 55]. It has already been reported that IL-21 alone or in combination with IL-15 could influence the frequency of memory $CD8^+$ T cells. In some cases, the proliferation resulted from the cytokine combination can be used to induce protection to virus infection or tumor using association with specific antigens [22, 26, 56]. When we compared the MFI of CD44 expression on CD90.2+CD8+ population (Figure 6B) with the numbers of CD44high Tet+ cells in a CD90.2+ population (Figure 6D), we could see that some of the groups with higher CD44+ MFI [pIL-21(37.24), pIL-21+pIL-15 (33.85) and pIL-21+pIL-15+pgB (34.15)] also differed more from the vector [pIL-21($p<0,01$), pIL-21+pIL-15 ($p<0,01$), pIL-21+pIL-15+pgB ($p<0,001$). pIL-21 group presented a high MFI (38.88), but the SD was 3.01.

To develop viral vaccines or therapies for protection from viral infection and replication, CD8+ T cell function should be considered. In case of HSV-1, $CD8^+$ T cells play an important function on herpes recurrence [7, 57-59]. We tested the function of $CD8^+$ T cells from each plasmid treatment by determining IFN-γ production by these cells after antigen stimuli. All plasmid treatments lead to an increase in magnitude of $CD8^+IFN\text{-}\gamma^+$ cell, compared to vector injection alone (Figure 6E). However, the compensation for $CD8^+IFN\text{-}\gamma^+$ cell frequency by plasmid cytokine treatment was most evident when pIL-21, pIL-15 and pgB were administered together. This observation is easily revealed when cell numbers were analyzed. Interleukin-15 signaling is important for memory $CD8^+$ T cell proliferation, but for IFN-γ production and cytotoxity of this cells, others immune factors can influence for better response [60, 61]. Priming of CD8+ T cell required MHC-I: TCR and CD28:B7 interaction and also $CD4^+$ T cell help is needed. In case of memory T cells is not clear if these three signals are crucial events [62].

Bolesta et al. showed that the combination of plasmids coding for IL-21 and IL-15 can lead to an optimal augmentation of HIV specific T cell response when provided after Ag priming. They defend the notion that IL-21 exposure to an immune system that has been primed with an antigen specific stimulation may lead to the optimal augmentation of immune response and also that IL-21 and IL-15 combination can replace the CD4+ T cell help for memory cells [56]. Also, protective immune responses were observed in tumor and viral models after IL-15 and IL-21 gene delivery, and this protection was related with substitution of CD4+T cell help by the cytokines [45, 50]. Our results suggest that pIL-21 and pIL-15 plasmid treatment can provide additional signals to memory CD8+T cells to proliferate by homeostatic ways and the antigen provide a signal that potentiates IFN-γ production. Studies with naive T cells have shown that IL-21 slightly increases IL-15 induced proliferation of CD8+ T cells, but IL-21 can prevent the CD28 down-regulation mediated by IL-15. Interleukin-21 can preserve the capacity of naive CD8+ T cells to be activated upon IL-15 stimulation [63]. Perhaps when pIL-21, pIL-15 and pgB as antigen was associated, the maximal signals necessary for memory CD8 proliferation and function are provided.

An important point to be considered is that all of these studies reported previously used cytokine treatment during the priming phase, together with antigen, and analyzed what happened in the memory phase. However, in our system, the antigen-specific population was expanded during priming phase with viral infection and the cytokine plasmids were administrated after the memory population was established. Our data suggested that during the memory phase, IL-21 and/or IL-15 could support the homeostatic proliferation of antigen specific $CD8^+$T cells and that antigen was not crucial for this process. Indeed, the critical moment for the

generation of an efficient memory T cell pool to viral infection appears to be during the initial antigen encounter, rather than during memory. Initial contact with antigen can impact the magnitude and quality of initial cytotoxic T lymphocytes responses as well as the efficacy and longevity of the ensuing $CD8^+$ memory pool [10, 64]. We must also consider that HSV-1 causes a latency infection and that low levels of antigen expression during latency can play some effects on $CD8^+$ T cells signaling in vivo.

Taken together, all results suggest that during the priming phase, TCR signaling provided by anti-CD3 *in vitro* or virus infection *in vivo* is crucial for the magnitude of $CD8^+$ proliferation and function, and that cytokines improve the response. During memory, cytokine signaling provides some additional signal that can overcome the necessity of antigen. To better understand the action of these $CD8^+$ T cells generated in this system during an infection *in vivo*, a study of protection against HSV needs to be performed. We are currently doing these studies.

In summary, we propose that IL-21 and IL-15 can be used as genetic adjuvants in cell therapy and vaccination to improve anti-viral immunity. These cytokines can induce proliferation of naive and memory $CD8^+$ T cells without need for antigen and the combination of IL-21, IL-15 and antigen was more effective for the HSV-1 vaccine scheme proposed. The optimized genetic system developed in this study is highly cost-effective, and presents the same effects described before when the purified cytokines were employed. Finally, our data suggest that if the memory pool is expanded by IL-21 and IL-15, contacts with antigen such as infectious challenge will provide the signals for IFN-γ production by antigen specific $CD8^+$ T cells, thus enhancing the response.

Legends

Figure 1: *In vitro and in vivo* expression of pIL-21 and pIL-15 plasmid constructions: For *in vitro* analysis expression levels of plasmid construct were assessed using transiently transfected H293 cells as described in materials and methods. Briefly, cells were plated in six well tissue culture dishes at a density of 2 x 10^5 cells per well in complete DMEM plus 10% FBS and allowed to adhere overnight. The next day cells were transfected with either vector or plasmid constructions (1μg/well) using lipofectamine 2000. After forty-eight hours, cell supernatants were harvest and analyzed for the presence of IL-21 protein by ELISA. For in vivo analysis mice were injected in tibial muscle with 75μg of each cytokine plasmid or control vector. Three days after DNA injection the muscle were excised, misled and cultured in RPMI with 10% FBS for three more days and the cytokine levels in the supernatant was measured by ELISA A) Expression level of pIL-21 into pVIVO-2 under control of Ferritin promoter and under control of CMV promoter in vitro,. B) Expression level of pIL-15 into pVIVO-2 under control of Ferritin promoter and under control of CMV promoter C) Expression level of pIL-21 into pVIVO-2 in vivo (Results of three independent experiments; three mice per group) (*, $p<0.05$; ** $p<0.005$)

Figure 2: *In vitro* activity of IL-21. Supernatant from H293 cells transfected with pIL-21 or vector was used to supplement RPMI 1640 medium plus IL-2 and used to culture splenocytes from C57BL/6. Six days after culture the cells were counted stained with anti-CD3 FITC and anti-$CD8^+$ Cy.Chrom and analyzed by flow cytometry. A) Dot plot with percentage of $CD3^+CD8^+$ cells recovered after treatment with hIL-2; B) Dot plot with percentage of $CD3^+CD8^+$ cells recovered after treatment with hIL-2 (50U/well) plus vector supernatant; C) Dot plot with percentage of CD3+CD8+ cells recovered after treatment with pIL-21H293 supernatant (40ng/mL) plus hIL-2 (50U/well); D) Dot plot with percentage of CD3+CD8+ cells recovered after treatment with recombinant IL-21 (R&D) plus hIL-2 (50U/well); E) Number of CD3+/CD8+ cells recovered after stimulation with anti-CD3 (0.25μg/mL) in the presence of mediums containing hIL-2 (50U/well), vector supernatant, pIL-21H293 supernatant (40ng/mL) or recombinant IL-21 positive control (40ng/mL) for three

days after culture. F) pIL-21H293 supernatant was treated with anti-IL-21 (15, 25 or 50 μg) for one hour and used to culture the total splenocytes like previous experiment. (*, $p<0.05$; ** $p<0.005$).

Figure 3: *In vitro* activity of IL-15: IL-15 bioactivity in supernatants of cells transfected with pIL-15 expression plasmid. Briefly, H293 cells were transfected with pIL-15 expression construct or vector pVIVO-2, and the supernatant were collected 72h after transfection, concentrated with centricon-3 and the amount of IL-15 determined by ELISA. The supernatants were diluted and tested by CTLL-2 bioassay to measure IL-15 bioactivity. Commercial IL-15 (2,5, 1,25 and 0,62μg) was used as a positive control. The experiment was repeated multiple times with similar outcomes. The data represents results from one such experiment. IL-15 supernatant (open circle), positive control (filled square), vector supernatant (asterisk).

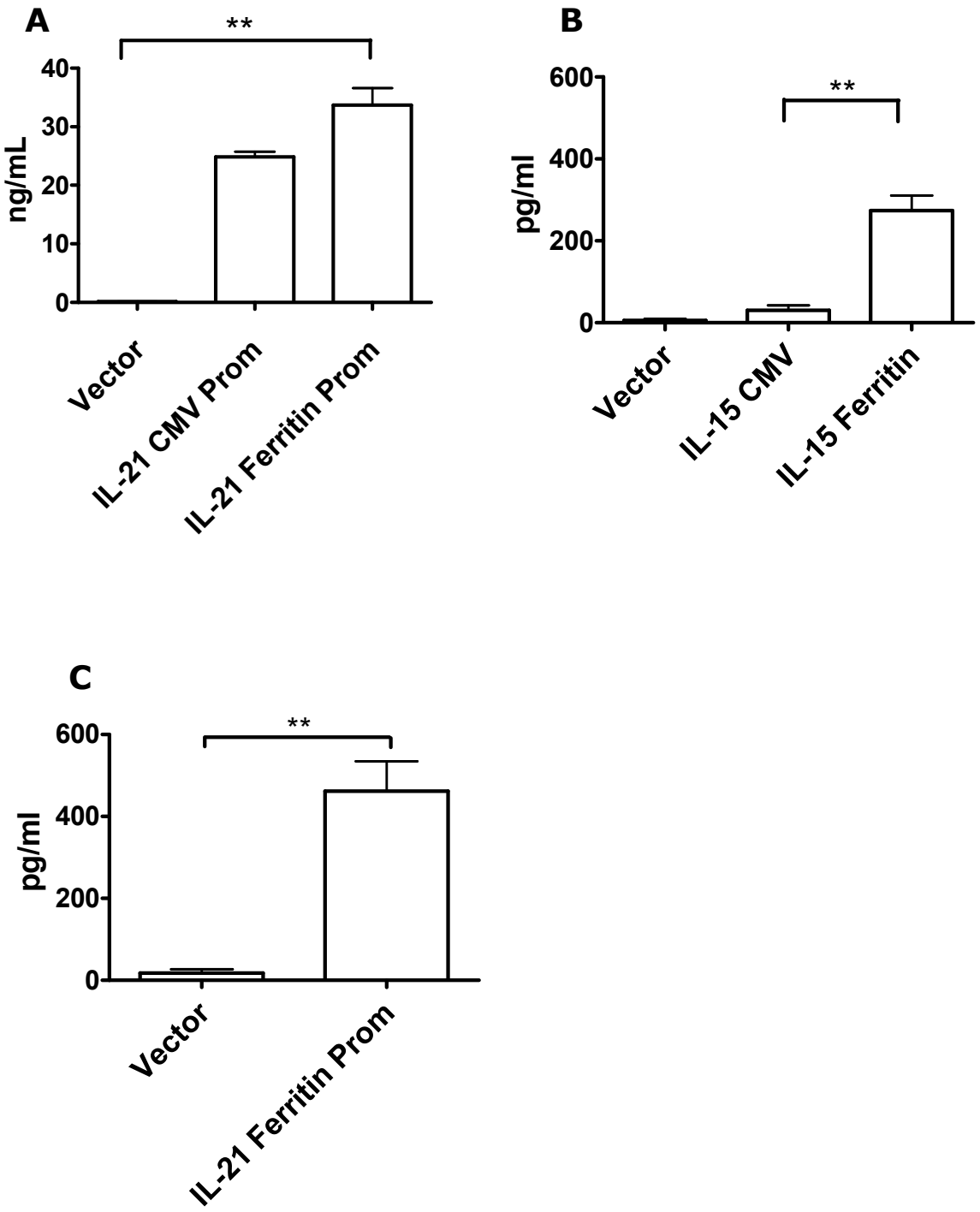
Figure 4 - *In vivo* activity of pIL-21 and pIL-15 constructions over $CD8^+$T cell expansion- effector phase. Female C57BL/6 was infected intraperitoneally with 5 $x10^4$ *PFU* of HSV-KOS. Two days after the mice were treated by intramuscular injection with 75 μg of pIL-21, pIL-15 or vector. Twelve days after infection mice were sacrificed, splenocyte isolalated and RBC lysed. Cells were incubated in 37^0C/5% CO_2 for 5 hours with SSIEFARL peptide and golgi-plug. After five hours the cells were washed and stained with IFN-γ-intracellular (PE) and $CD8^+$ (FITC). FACS plots with percentage of $CD8^+IFN\text{-}\gamma^+$ cells, A) uninfected; B) infected and vector treated; C) infected and pIL-21 treated; D) infected and pIL-15 treated. E) Absolute numbers of $CD8^+IFN\text{-}\gamma^+$ cells of mice uninfected, vector treated or pIL-21 treated; F) Absolute numbers number of $CD8^+IFN\text{-}\gamma^+$ cells of mice uninfected, vector treated or pIL-15 treated (Four mice per group). The experiment was repeated three times and the pattern of results were the same. The figure represents one such experiment. *(*,P<0.05; **P<0.001;***P<0.0001)*

Figure 5: Expansion of SSIEFARL $CD8^+$ T cells with pIL-21 and pIL-15.
A) Titration of total $CD90.2^+$ splenocytes from SSIEFARL transgenic mice needed for adoptive transfer. Two female C57BL/6 phenotype $CD90.2^+$ SSIEFARL transgenic were sacrificed and splenocytes isolated and pooled. Splenocytes were stained with

CFSE and transferred into female C57BL/6 phenotype CD90.1^{+} through the tail vain. Each group of three mice received different amounts of total splenocytes CD90.2^{+}/SSIEFARL that contains decreasing numbers of CD90.2^{+} SSIEFARL CD8^{+} cells (1.44 x 10^{5}; 2.88 x 10^{4}; 2.88 x 10^{3} or 2.88 x10^{2}). Twenty four hours after transfer the mice were infected intraperitoneally with 5 x10^{6} *PFU* of HSV KOS. The mice were killed seven days after infection, splenocytes were isolated, red cell lysed with RBC and the cells were stained with SSIEFARL tetramer PE; CD90.2 APC and CD8^{+} Cy-Chrom and analyzed by FACS. *(***, p<0.001*)

Figure 6: pIL-21 and pIL-15 induces HSV-1 specific memory CD8^{+} T cell expansion. A) C57BL/6 CD90.1^{+} mice were adoptive transferred with 2 x 10^{4} splenocytes from SSIEFARL CD90.2^{+} mice and infected intraperitoneally with 5 x 10^{6} PFU of HSV-KOS. Sixty days after infection, groups of four mice were treated with 75μg of pIL-21, pIL-15, pgB and vector, alone or in combination. The immune response in spleen was analyzed 10 days after plasmid treatment. B) MFI of CD44 on CD90.2+CD8+SSIEAFARL+ population gated on CD44 specific cells recovered from each group of mice. Gray line: vector only, black line: cytokine encoding plasmid. Results are expressed as a mean fluorescence intensity (MFI); C) Absolute numbers of CD90.2+ CD8+ cells recovered from spleens of each group of DNA treatmen. D) Number of CD44^{high}Tet^{+} within a population of CD90.2^{+} cells recovered from spleen of each group. E) Number of CD8^{+}IFNγ^{+} cells recovered from spleens of each group of mice. Results are representative of four mice per group. *(*,P<0.05; **P<0.001;***P<0.0001)*. The experiment itself was repeated with same outcome. The analysis represents each cytokine encoding group compared with the vector group.

Figure 1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20
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Vector
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IL-21 Ferritin Prom
B
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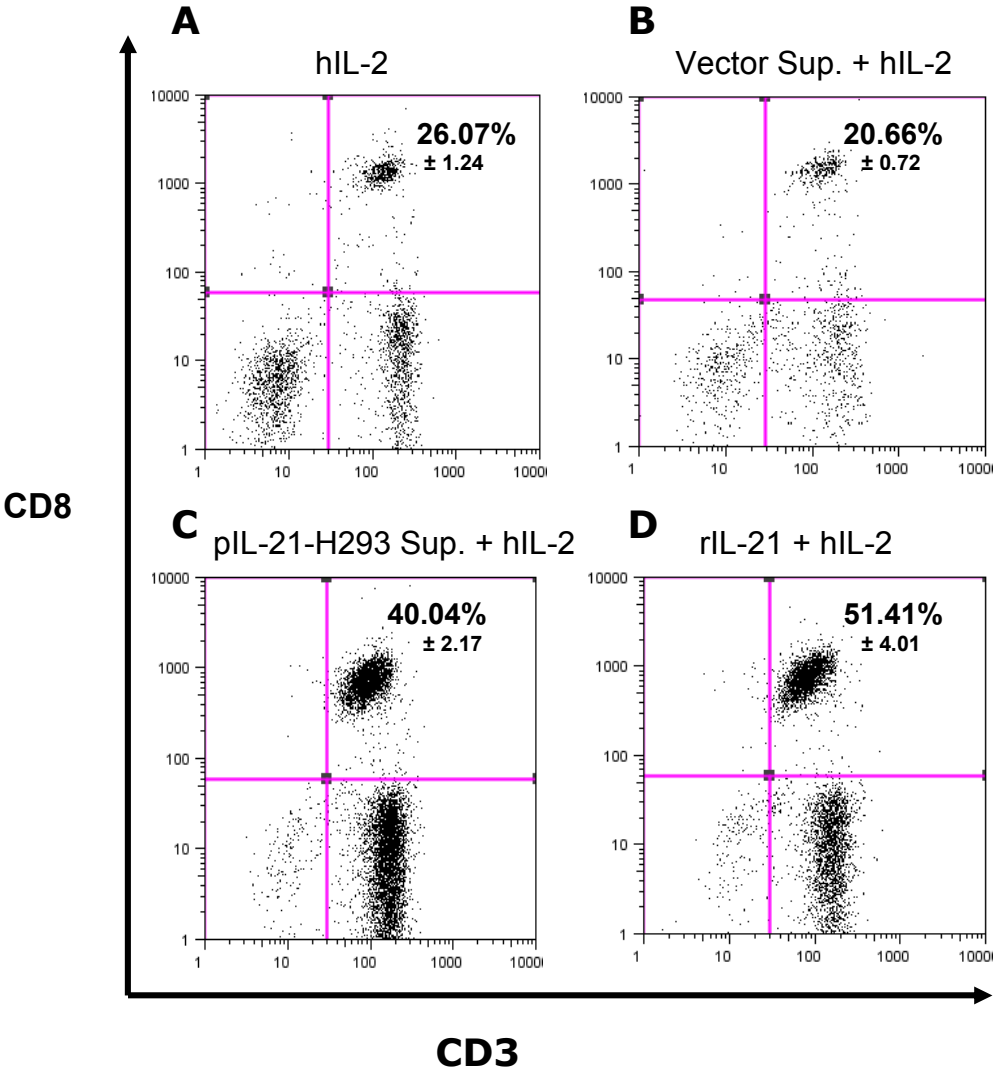
Figure 2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C
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± 4.01
CD8
CD3

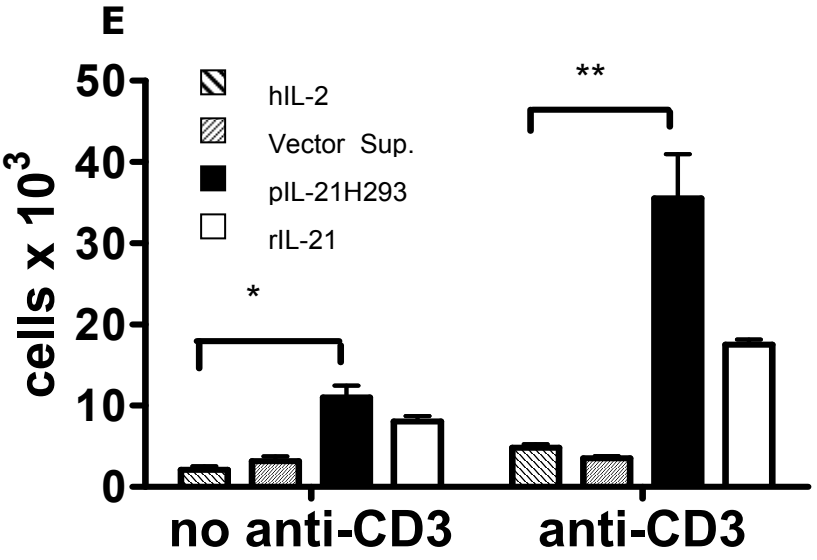
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Figure 2 (continuation)

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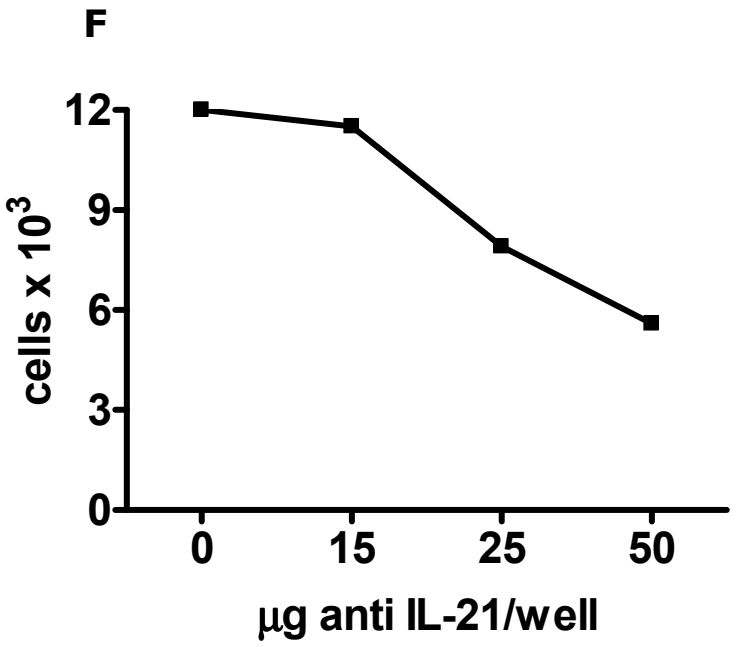


Figure 3

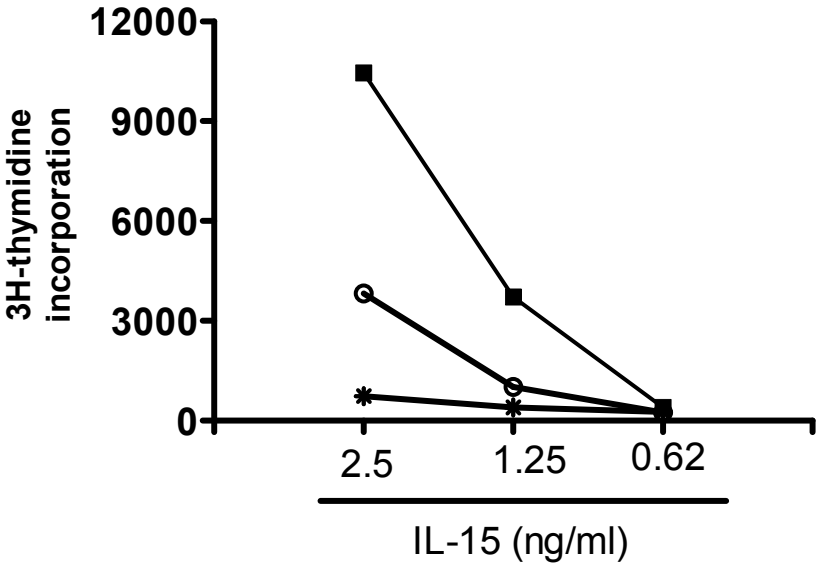


Figure 4

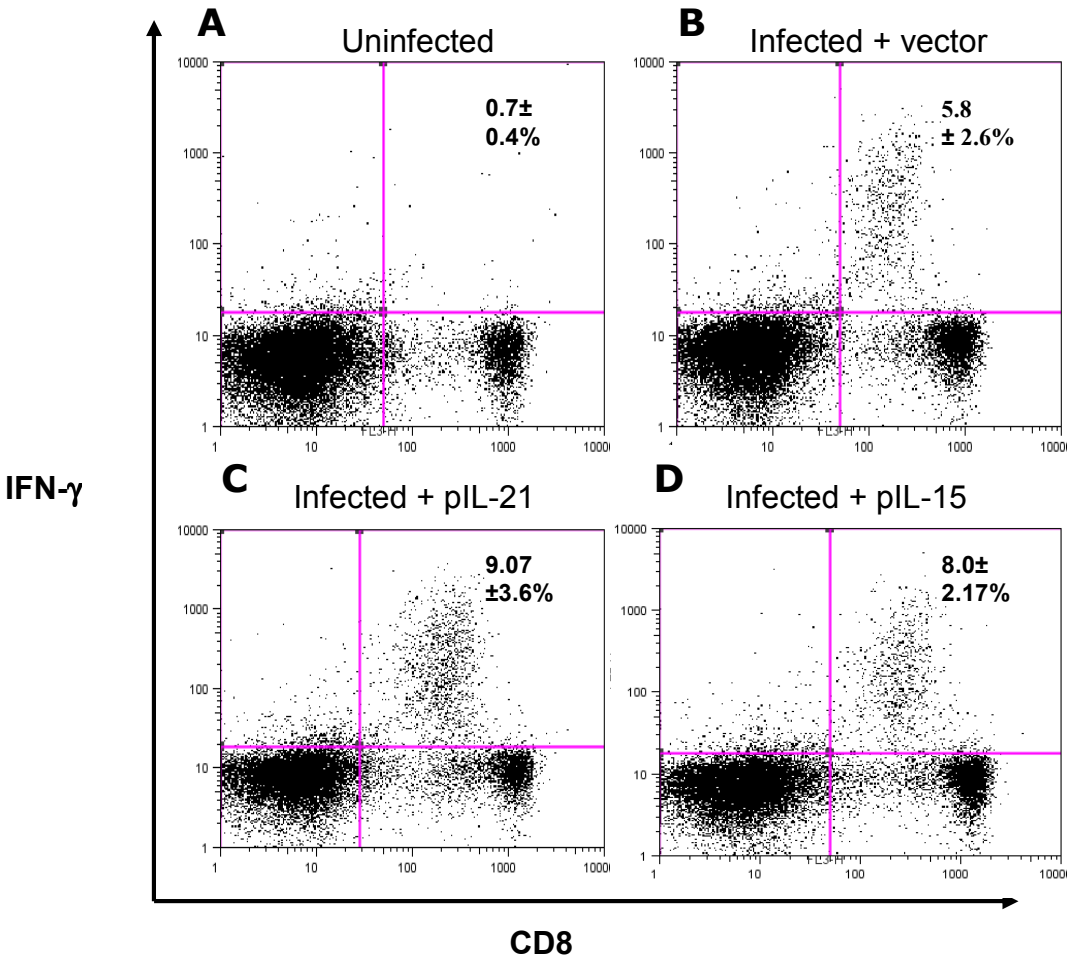
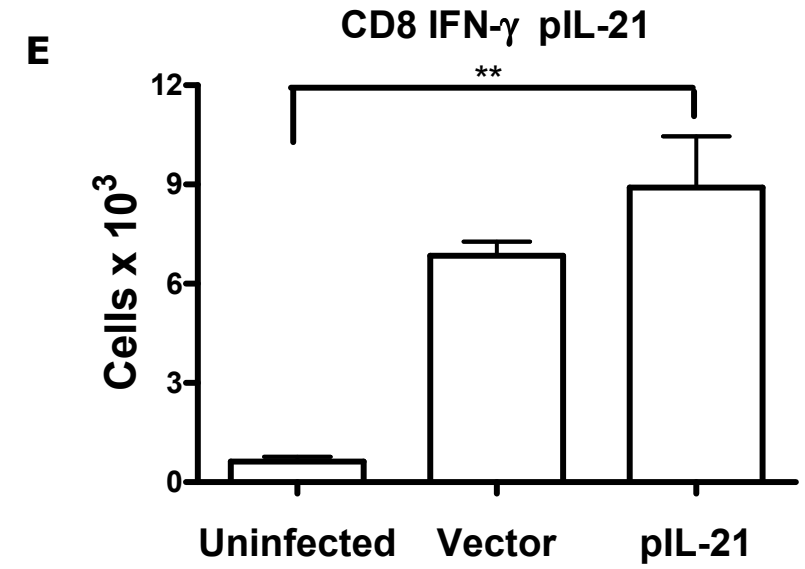

A
Uninfected
0.7±
0.4%
B
Infected + vector
5.8
± 2.6%
C
Infected + pIL-21
9.07
±3.6%
D
Infected + pIL-15
8.0±
2.17%
IFN-γ
CD8

Figure 4 (continuation)



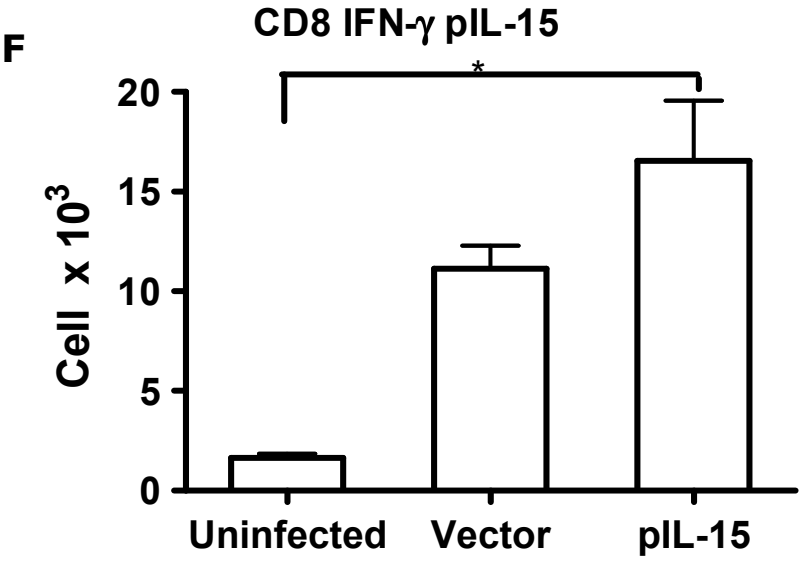


Figure 5

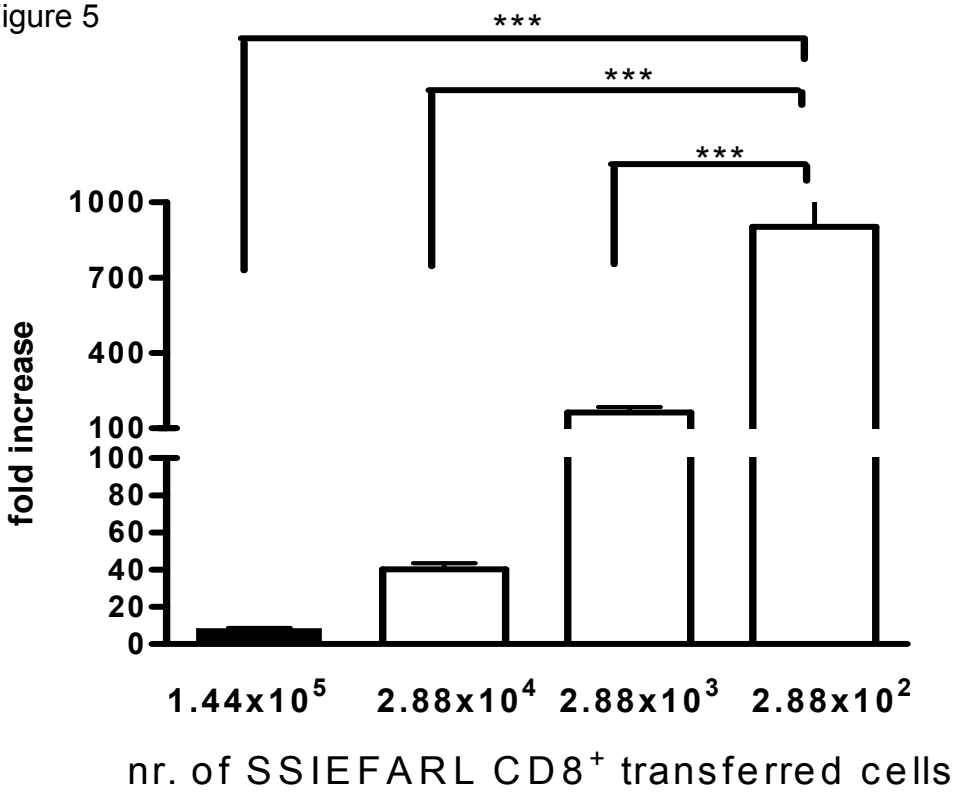


Figure 6

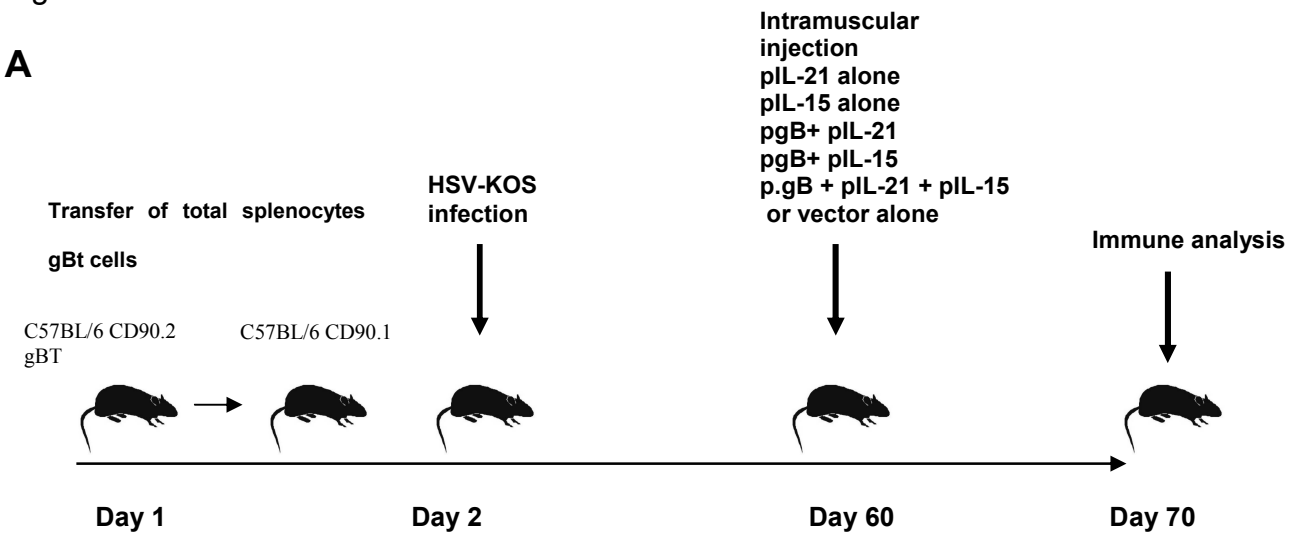


Figure 6 (continuation)

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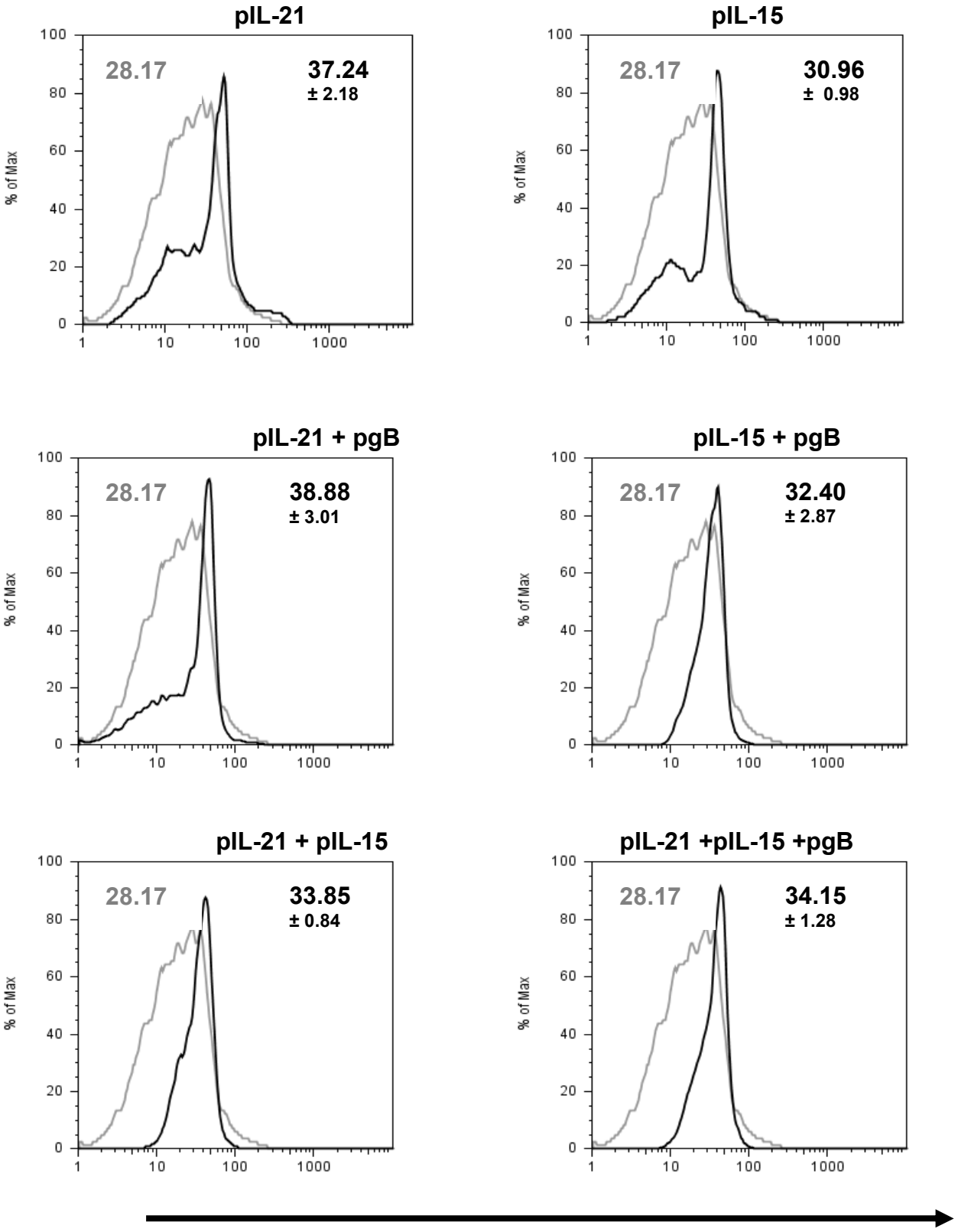
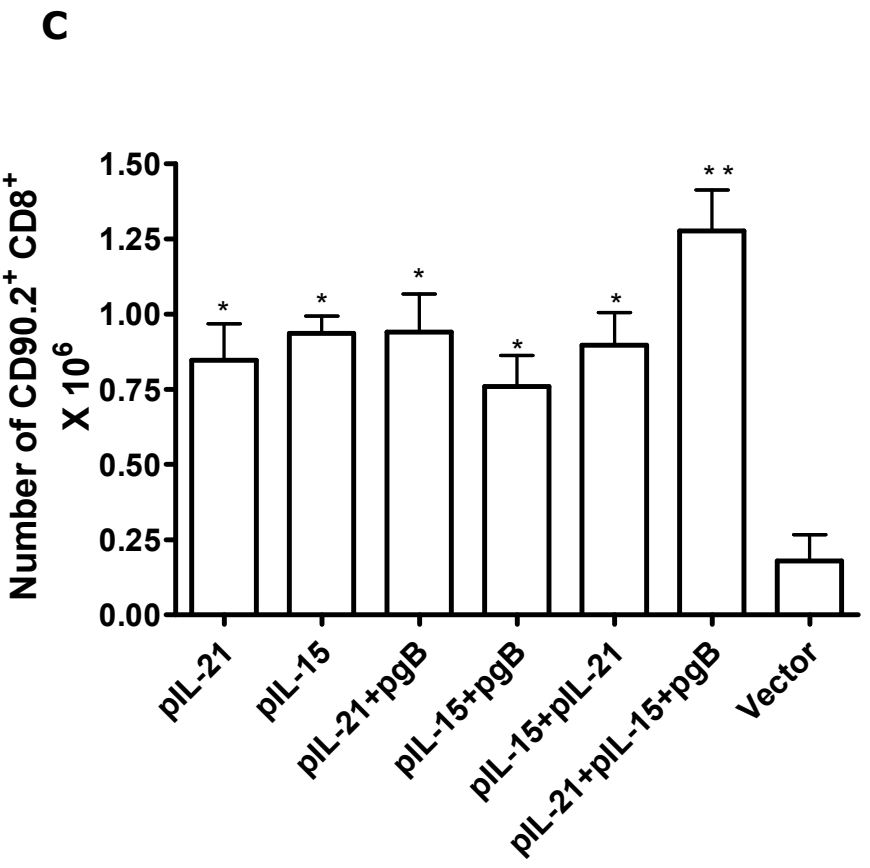
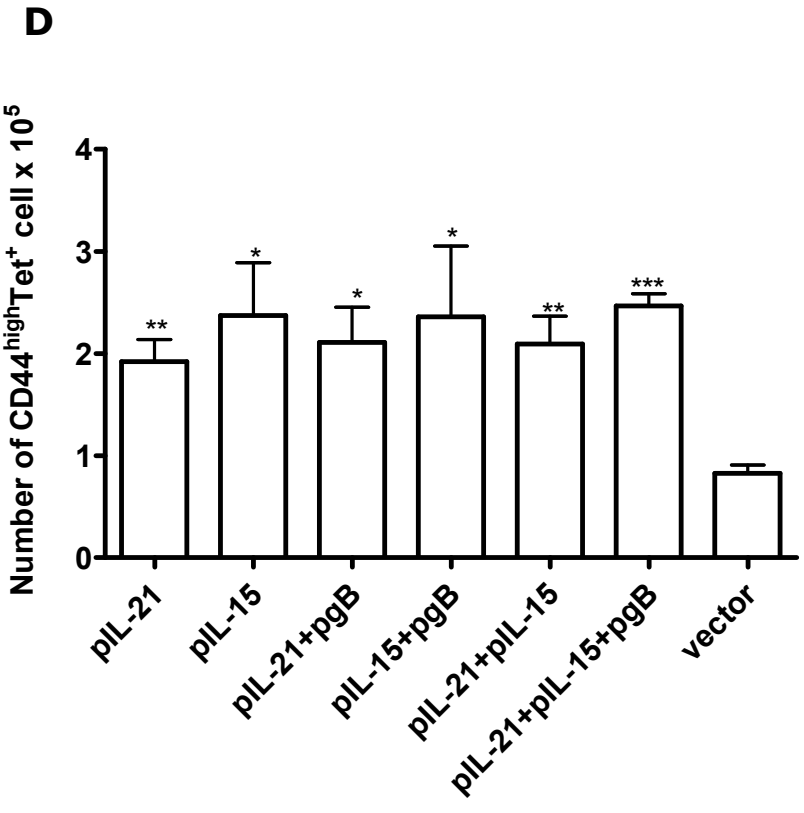


Population of CD90.2^{+} CD8^{+} SSIEFARL, gated on CD44^{+}

Figure 6 (continuation)





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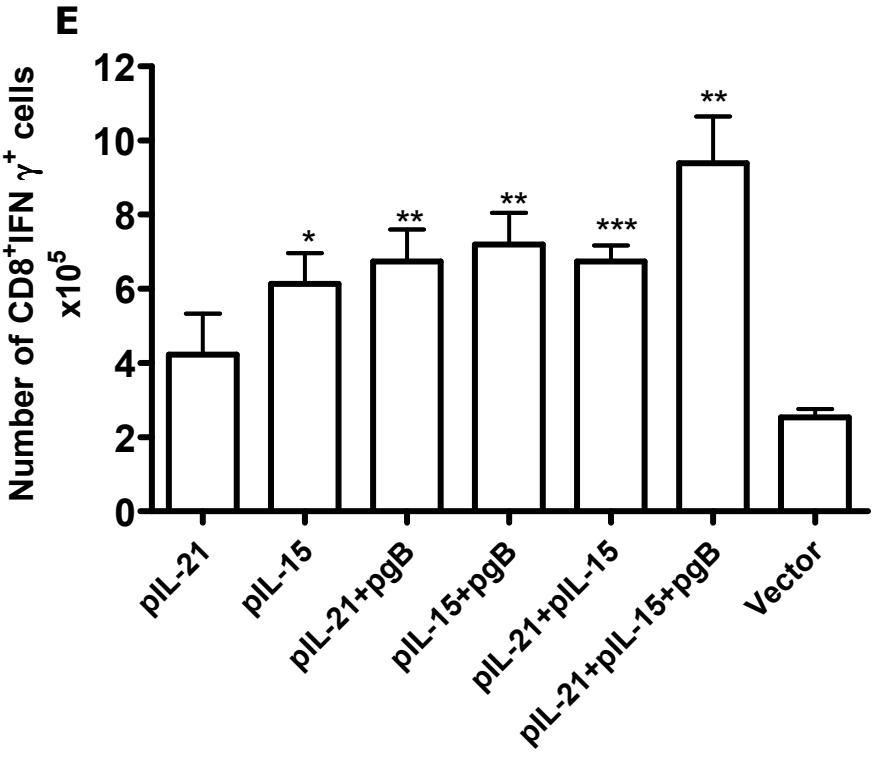

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CAPÍTULO III

Role of Interleukin-15 and Interleukin-21 in viral immunity: applications for vaccines and therapies.

Revista: Expert Review Vaccines

Role of Interleukin-15 and Interleukin-21 in viral immunity: applications for vaccines and therapies

Luiz Rodrigues & Cristina Bonorino

Keywords: Cytokines, virus, adjuvant, vaccine, memory CD8+ T cells.

SUMMARY

Everyone has at least one viral infection during their life, some more virulent and aggressive than others. The aggressiveness and progression of viral diseases depends on the type of virus and quality of antiviral response generated during innate immunity and maintained during adaptive immunity. Two recently discovered cytokines (interleukin 15 and 21) appear to be key regulators in this process. IL-15 induces an antiviral state during innate immunity through regulation of IFNα/β production and NK cell proliferation. During memory phase, antigen specific CD8+ T cells are highly dependent on IL-15 signaling. Interleukin-21 induces NK cell maturation and IFN-γ production and acts enhancing the proliferation of memory CD8+ T cells, its effects being pronounced when combined with IL-15. This review describes mechanisms and potential uses of these cytokines in the design of anti-viral vaccines and therapies.

Introduction

The common gamma chain (γc) cytokine family

The main goal of viral vaccines and anti-viral therapies is the induction of a potent and lasting antigen specific T cell response. Important tools in this process are factors that can influence T cell biology, such as cytokines. The common gamma chain (γc) cytokine family comprises IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. These cytokines signal cells through a heterodimeric complex receptor, and the γ chain of this receptor is the common receptor subunit for these cytokines[1]. IL-4, IL-7, IL-9 and IL-21 utilize IL-4Rα, IL-7Rα, IL-9Rα and IL-21Rα respectively as a specific receptor subunit[2-4]. IL-2 and IL-15 share the IL-2/15Rβ subunit besides a specific IL-2Rα and IL-15Rα and gamma chain [5]. Most of these γc family cytokines are involved in T, NK and B cell homeostasis and proliferation, and the lack of the γc gene results in severe immunodeficiency [6]. Because of that, several studies have investigated the use of these cytokines to improve immune responses to bacteria, parasites [7] or viruses [8,9], as well as against tumors [10]. One important reason to use these γc cytokine family members is that they regulate the development and magnitude of proliferation of immune cells. IL-2 controls CD4+ T cell survival and regulatory properties, IL-7 is important for thymocyte development [11], IL-15 regulates mainly the numbers and function of CD8+ T cells and NK cells [12], IL-21 controls T and B lymphocytes function and also acts in the transition from innate to adaptive immunity [13]. Another important reason is their role in memory development. Antigen specific memory CD8+ T cells and polyclonal memory CD8+ T cells do not efficiently proliferate in the absence of IL-15, protection being reduced [14,15]. Although IL-7 works as a survival signal for memory CD4+ T cells [16], during the early phase of an infection, effector CD8+ T cells that express high levels of IL-7Rα are highly commited to become memory cells and are also partially dependent on IL-15 for survival [17]. IL-21 produced by CD4+ T cells can provide the third

signal to naive CD8+ T cells, leading to their differentiation in response to antigen and costimulation [18]. During memory phase, IL-21 induces up-regulation of CD28 on memory CD8+ T cells, increasing the activation capacity of to be activated upon second challenge [19].

The anti viral response

A major strategy to eliminate viral infections is the induction of type I (α/β) inferferons. They are responsible for blocking replication of many types of viruses during acute and chronic phase [20]. Type I interferons also stimulates dendritic cells (DC) function as well as NK cell cytotoxic activity and IFNγ production [21].

CD4+ T cells provide help and restrict viral replication by secretion of cytokines, especially IFN-γ [22,23]. However, it is already well studied and proved that the effector response to clear viral infection during adaptive immunity is the cytotoxic T lymphocyte response (CTL), mediated by antigen specific CD8+ T cells [24]. The cytolytic function and proliferation of these cells is controlled by several different cytokines [25]. Finally, there are early observations from Jenner´s reports that several people don't die from smallpox infection because they develop, among other cells, antibody producing B cells after the immunization event [26], and consequently the contribution of antibodies in anti-viral protection cannot be underestimated.

IL-15 and IL-21 in the anti-viral response

Increasing evidence implicates IL-15 and IL-21 as new and important tools for the design of vaccines for viral infections. Several studies have successfully used IL-15 to induce type I interferons and promote an efficient antiviral response [27]. Secretion of type I interferons is controlled mainly by IL-15 through IFN-stimulated genes [28]. Also, IL-15

controls proliferation and IFN-γ secretion by NK cells during an acute viral infection [29,30]. IL-15 is also important in adaptive responses against viruses. Antiviral CD4+ memory T cells are partially dependent on IL-15 signaling [31]. IL-15 induces the survival, proliferation and cytolytic function of effector and memory anti-viral CD8+T cells [32,33].

Like IL-15, IL-21 can act on the initial phase of immune responses, conferring a functional mature phenotype to NK cells both in vitro and in vivo, and increasing the killing of virally infected cells [34,35]. However, most of its known effects are on adaptive immunity. During the priming phase of immune responses, IL-21 produced by CD4+ T cells has a direct effect on DC, leading to their activation and consequently the induction of effector T cell function [36]. This step is crucial for anti- viral immunity, because some types of viruses can only be cleared by an antigen specific immune response. Interleukin-21 controls T cell effector functions by autocrine and paracrine action on CD4+ and CD8+ T cells, respectively [37-40]. It also helps to control the magnitude of CD8+ T cell responses during a viral infection, or antigen administration [41]. Finally, IL-21, along with IL-4, significantly regulates B cell function. Mice lacking IL-21R have an impaired IgG production against different antigens [42].

Because γ-chain family cytokines play a crucial role in different phases of antiviral immunity, as well as in differentiation of CD4+ and CD8+ T cell after viral encounter, they have been widely used as vaccine adjutants. This review describes the role of IL-15 and IL-21 as molecular adjutants to viral vaccines. We present the antiviral mechanisms affected by each cytokine, and discuss how they can be applied to different types of viral vaccine.

IL-15 and Il-21: Biology

IL-15 is a cell associated cytokine that controls immune cell function

IL-15 is a pleiotropic cytokine that displays many immunomodulatory activities. It is mainly produced by macrophages and DC, its activities partially overlapping with those of IL-2 [43,44]. IL-15 can control NK cell proliferation and function, naive and memory CD8+ T cell proliferation and also the inflammatory response mediated by DC and macrophages [29,45,46]. The message for this cytokine is expressed by several tissues, such as placenta, skeletal muscle, monocytes and macrophages [47]. Although all these cells express the message for IL-15, its production and secretion are tightly regulated by alternative splicing both in mice and humans [48,49]. Two signal peptide IL-15 isoforms, a short one and a long one, are produced by alternative splicing [48]. They are poorly secreted and the short signal peptide isoform controls the production of the long peptide isoform through biding to the IL-15 promoter [50]. A very interesting mechanism involved in IL-15 signaling identified by Dubois and cols and dissected by others is the recycling of IL-15Rα and transpresentation to neighboring cells. In this system, IL-15Rα mediates a trans-endosomal recycling of IL-15, thereby leading to the persistence of IL-15 signaling even after withdrawal of this cytokine (Figure 1). This mechanism guarantees that even in low abundance of IL-15, memory CD8+ T cell numbers can be sustained, as well as NK cell proliferation and function [46,51-53]. IL-15Rα plays an important role in cellular mechanisms controlling IL-15 release and signaling. Soluble IL-15Rα expression is regulated by alternative splicing and proteolytic cleavage. There is evidence indicating that proteolysis could lead to an inhibitory sIL-15Rα:IL-15 complex, and that the balance between these two events would be controlled by viral or bacterial infection [54].

IL-15 plays a major role in DC function. DC is the major antigen presenting cell and stimulator of CD4+ and CD8+ T cells. Maturation of DC is necessary for activation of naive

T lymphocytes and is regulated by Toll like receptors (TLR) triggering and cytokines [55,56]. In viral infections, TLR-9 and TLR-3 can be triggered by CpG DNA and dsRNA respectively, inducing DC maturation [57]. During the innate immune response, high numbers of DC migrate to the site of infection and get activated [58]. To prevent excessive immune responses and unnecessary inflammation, DC undergoes apoptosis. Depending on the type of infection, reduction in DC numbers could compromise the immune response; however these cells can overcome apoptosis through up-regulation of IL-15 and IL-15Rα expression. This complex can mediate the survival of DC through induction of Bcl-2 expression, an anti-apoptotic molecule [51].

Indeed, IL-15 has a major role controlling DC function and activation of the immune system during intracellular infection, and this control involves IL-12 production. CpG DNA is released during bacterial or viral infection, binding to TLR9 in conventional DC (cDC), thus inducing IL-15 production and secretion. The IL-15 produced binds to IL-15Rα on the same cDC and induces the expression of CD40, which interacts with CD40L expressed on plasmacytoid DC (pDC). The interaction CD40 (cDC):CD40L (pDC) works as a signal to cDC produce IL-12. [59]. The biological relevance of this phenomenon is that the induction of inflammatory immune responses against viruses or other intracellular pathogen is mediated by cooperation of cDC and pDC and the crosstalk between these cells is controlled by IL-15 secretion and biding to its receptor [60].

Viral immunity mediated by IL-15 signaling

As previously discussed, type I IFN play an important role in innate antiviral immunity. These IFNs induce resistance to viral replication, increasing antigen presentation mediated by MHC class I and activating NK cells to kill infected cells [21,61]. Indeed. IFNα/β are the main immunomodulatory molecules produced early in viral infection and IL-

15 is one of the regulators of this phase. However, it is still unclear if IL-15 expression is only activated by type I IFN inducers or is also regulated by them.

NK cells and DC are the main innate immune cells involved in early antiviral response [62]. IL-15 is a stimulator of NK cell activity and mediates IFN-γ production and proliferation in vitro and in vivo [63]. In *Herpes Simplex* Virus-2 (HSV-2) infection, NK and NKT cells are the mainly source of IFN-γ, and its production is impaired in IL-15 knockout mice that succumb after intravaginal infection [64]. Recently, Lucas and cols proposed a complex mechanism where IL-15 controls NK cell priming. Their investigations showed that following a viral infection, DC undergo TLR stimulation that leads to type I IFN production, these molecules inducing the up-regulation of IL-15/IL-15Rα by DC. The cytokine/receptor complex trans-presented by DC is indispensable to prime NK cell effector function in secondary lymphoid organs [65]. The antiviral activity of IL-15 during innate immune response also was observed in macrophages, when these cells were stimulated with IL-15 and produced nitric-oxide helping to reduce poxvirus replication. This activity is also related to IFN-β up-regulation [66]. Thus, signaling through the IL-15 pathway is one of the key issues in early antiviral immunity. However, sometimes, the virus can bypass this phase and adaptive immunity is then needed.

During adaptive immunity the most effective cell to reduce viral load and replication is the cytotoxic CD8+ T cell [67,68]. These cells recognize viral peptides complexed with class I MHC molecules presented by infected cells. After peptide:MHC class I recognition, CD8+ T cells secrete perforines and granzymes that induce pore formation and lyse infected cells, also producing IFN-γ [69]. There are two classes of CD8+ T cells, effector CD8+ T cells and memory CD8+ T cells [70]. Memory CD8+ T cells proliferate and induce effectors functions faster and more efficiently than effector CD8+ T cells, and both classes are influenced by IL-15 for survival, proliferation and action [33,71].

A very important role of IL-15 to be considered in antiviral immunity was observed by Yajima e cols. They showed that IL-15 induces the up-regulation of anti-apoptotic molecules BCL-2 on both IL-7Rαlow and IL-7Rαhigh CD8+ T, regulating the contraction phase. According to them, IL-15 controls the number of cells that do not die and have a chance to become memory cells [72]. If we consider the transpresentation system in the contraction phase, after viral infection, DC up-regulates IL-15Rα and IL-15 and transpresent the complex to CD8+ T cells that express IL-2/IL-15Rβ as well as γ chain. IL-15 signals cells that express especially high levels IL-7Rα, up-regulates BLC-2 and survive, becoming memory cells with high antiviral capacity. For cells with a history of previous activation and replication, as antigen experienced CD8+ T cells, IL-15 appears to be a more effective survival factor than IL-7, a phenomenon that is partly related to their expression of cytokine receptors [73]. The clinical importance of the expression of IL-15Rα on CD8+ T cells during acute viral infection was demonstrated in mononucleosis, in people with primary Epstein-Barr virus (EBV) infection. The virus down-regulates the expression of IL-15Rα and the cell reduces the response to IL-15 [74].

After the early phase of viral infection, basically three patterns of viral infection can follow: viral clearance, viral latency or viral persistence [75,76]. An example of viral clearance is *Influezae* virus. In this case, a small pool of anti-specific memory CD4+ and CD8+ T cell are maintained by homeostatic proliferation driven by cytokines as IL-7 and IL-15 [77]. In case of latency, HSV-1 and 2 infection is the more investigated model, and different mechanisms for the maintenance of CD8+ T cells were described. HSV-1 establishes latency in the trigeminal ganglion after epithelial infection and antigen specific memory CD8+ T cell surround the neurons and express activated phenotype, releasing lytic granules and producing IFN-γ, thus maintaining the latency state [78]. IL-15 is the major cytokine to maintain the antigen-specific IL-7Rα+ CD8+ T cell alive, and theses cells are the mainly

candidates to become a memory population that will surround the neuron [79]. Finally, in case of persistent infection, CD8+ T cells receive continuous TCR stimulation as a result of high levels of virus that normally result in T cell exhaustion [80,81]. Cytokines do not influence significantly the CD8+ T cell proliferation in persistent infection [82].

Memory CD8+ T cells depend heavily on IL-15 for proliferation and survival, and IL-15-/- mice are completely deficient of memory CD8+ T cells [5]. After *Influenzae* virus infection, memory CD8+ T cells in pulmonary parenchyma lose IL-15R, driven by the virus and become less susceptible to homeostatic proliferation, disappearing during the time. Thus, all these observations seem to indicate that IL-15 is crucial for maintaining basal T cell homeostatic turnover, and IL-15:IL-15Rα contributes to the long survival of memory CD8+ T cell [52], that directly controls the viral replication in vivo.

IL-21 is a key factor that controls transition from innate to adaptive immune response

IL-21 is the most recently described member of common γc receptor family. IL-21 was identified as a ligand for the orphan receptor that had properties of inducing proliferation of Baf3 cells transfected with this receptor and cultured with supernatant of T lymphocytes activated with anti-CD3. This interleukin was first showed as a product of activated CD4+ T cells[83], however recent studies demonstrated that NKT cells can also produce IL-21 [84]. The IL-21R is expressed by CD4+ T cells, CD8+ T cells, NK cells[83], B cells [85] and DC [36] and keratinocytes [86]. The expression of IL-21R is up-regulated after TCR stimulation [87].

DC generated in the presence of IL-21 was first characterized by reduced MHC II expression, high antigen uptake and low stimulation capacity for T cell activation. IL-21 inhibits DC activation and maturation after stimulation by lipopolissaracharide [36]. Also

during innate immune responds, IL-21 decreases NK cell expansion, and induces these cells to produce more IFN-γ , increasing their cytotoxic activity [13]. This induction of NK maturation and function was observed in human [88] as well as in mice cells (Brady, 2006). Transcription of IFN-γ, IL-12R, T.bet and IL-18R genes are up-regulated on IL-21 treated NK and T cells [89]. This observation suggest that IL-21 can down-regulate some innate immune functions, providing a signal of the initiation of adaptive immunity.

The role of IL-21 as a co-activator of CD4+ T cells is still not clear. Most of the studies indicate a major importance for IL-21 on CD8+ T cell activation. Depending on the stimulation received, naive CD4+ T cell can follow three different routes: Th1 polarization (inflammatory), Th2 polarization (anti-inflammatory) or Th17 polarization (neutrophil differentiation and infiltration during infection) polarization [90,91]. IL-21 is produced predominantly by Th2 cells and inhibits the differentiation of naive T cells into Th1 cells in vitro [92]. This is regulated by repression of Eomes, an IFN- inducer. However, once the cells are differentiated into either Th1, Th2 or Th17 lineage, IL-21 doesn't have any effect on IFN-γ production [93]. Th17 also was characterized as an IL-21 producer, and its effects in autoimmunity is regulated by autocrine action of IL-21[94].

The proliferation of CD8+T cells is highly affected by IL-21 [39,40,95]. Actually, IL-21 was discovered as a co-mitogen to augment T cell proliferation [83]. Application of IL-21 to expand CD8+ T cells was used in viral [96], tumor [97], bacterial [98] and parasite models. During priming of naive CD8+ T cell, engagement of the TCR and CD28 stimulates the cells to enter cell cycle and undergo differentiation, proliferation and effector function [99,100]. DC can provide the two first signals, MHC and B7 [101]. IL-21 is the factor released by CD4+ T cells that drives an effector phenotype, working as an additional signal [18].

IL-21 is especially effective in CD8+ T cell proliferation when associated with IL-15 [95]. Combination of IL-21 and IL-15 can influence naive and memory CD8+ T cell

proliferation and function in vitro and in vivo. Microarray analyses demonstrated that this combination induces the up-regulation of granzime A, granzime B, *bcl*-2 and IL-7R genes [102]. This synergism was also evaluated at the level of cell signaling, the proliferation of memory and naive CD8+ T cells induced by IL-15 plus IL-21 being controlled by suppressor of cytokine signaling 1 (SOCS1) [103]. Another important finding by Alves and cols was that memory CD8+ T cells proliferate in the presence of IL-15, but this cytokine induces down-regulation of CD28 reducing the activation capacity of these cells, however, in presence of IL-21, CD28 expression is up-regulated and the cells respond better to a second antigen stimulation [19].

Il-21 action during viral infection

The mechanistic role of IL-21 during viral infection has been poorly investigated. The majority of studies are related with the synergism of IL-21 and other γ-chain cytokines to improve anti-viral immune response. However, some data reported on effector and memory CD8+ T cells suggest some specific actions of IL-21 during viral infection.

It is postulated that viruses might induce phenotypically distinct T cells. Cells with specificities for different persistent viruses vary in phenotype and function. For instance, EBV-specific T cells are predominantly $CD45R0^{+}CD28^{+}CD27^{+}$, and in asymptomatic HIV-carriers, HIV-specific T cells are mostly $CD45R0^{+}CD28^{-}CD27^{+}$, contrasting the phenotype of CMV-reactive T cells that are predominantly $CD45RA^{+}$CD27+[68]. CD27 and CD28 are markers involved on T cell proliferation and activation, receptively [104], CD27-deficient mice have a reduced ability to form adequate numbers of Ag-specific T cells upon viral infection [105]. IL-2 and IL-15 induces expansion of $CD27^{-}$ virus-specific T cells, whereas IL-21 promoted outgrowth of $CD27^{+}$ cells in CMV- infection model [106]. So IL-21 controls the expression of CD27 in a population of viral specific effector CD8+ T cell, guarantying the

proliferation and expansion properties. As discussed before, IL-21 rescues the CD28 expression, abolished by IL-15. Many virus establish latency on non-lymphoid organs, surrounded by endothelial cells, CMV and HSV-1 and 2 are example [107] upon reactivation antigen-specific memory CD8+ T cell need to migrate to lymphoid organs to get additional signals [108]. These cells proliferate by action of IL-15 produced by endothelial cells but they can not re-express CCR7 receptor to migrate to lymph node without additional signal. IL-21 lead to the re-expression of CCR7 in antigen- CD8+ T lymphocytes driven the migration to lymph nodes, this was observed for CMV infection [41]. A diagram of this antiviral mechanism that can be proposed to IL-15 and IL-21 during reactivation of HSV-1 from latency can be seen on figure 2.

HIV infected CD8+ T cell demonstrated reduction on cytolitic function, but this cells can recover the expression of perforines and granzymes after culture with IL-21 [96]. Holm et al firstly demonstrated that one to three days after an HSV-2 infection CD4+ T cells up-regulates the IL-21 gene transcription and this is related with IFN-γ production as well [109]. Then IL-21 has some specifics actions during the anti-viral immunity programming and maintenance.

Applications of IL-15 and IL-21 in Viral Vaccines and Therapies.

With all the evidence on IL-15 and IL-21 activity during innate and adaptive immunity, it became evident that efficient vaccines and therapies could be proposed based on these cytokines. Several labs are trying to develop such antiviral vaccines.

An important observation for the application of these two cytokines as adjuvants in therapies and vaccines was their synergistic effect in innate as well as in adaptive immunity, reported by several studies. IL-15, in combination with IL-21, strongly enhanced IFN-γ production by primary NK cells and T cells during the early phase of viral infection, and this

effect is mediated by STAT4 [110]. On CD8+ T cells, the combination of IL-21 and IL-15 was superior to expand effector and memory cells compared to each cytokine alone. This expansion is accompanied by IFN-γ and granzyme B production and *c-jun* gene expression [102].

Therapies with cytokines are extremely expensive if recombinant proteins are used, especially because the dose administrated in vivo used to be high. An alternative methodology is DNA therapy, in which cytokines can be cloned into plasmids and injected as adjuvants [111]. DNA therapies are cost effective and technically easier, compared to recombinant protein production [112]. IL-21 and IL-15 DNA were tested in the same viral infection model fist time by Cui and cols, when they showed that pre-treatment with plasmid coding for IL-15 reduced in 40% (and IL-21 in 50%) the death caused by a lethal dose of HSV-1 [8]. The benefits of the synergistic effects of IL-15 and IL-21 as a plasmid administration were observed for an HIV-1 Env-specific DNA vaccine through induction of a durable Env-specific immune response. This response is based on effector and memory CD8+ T cell function and proliferation, and also protection against challenge with vaccinia virus expressing HIV-1 proteins [9].

Overexpression of IL-15 or administration of IL-15 (as a protein or expressed by plasmid DNA) augments protection of mice from a variety of infections [113,114]. The use of IL-15 has been explored in the development of anti-HIV therapies and vaccines. Stimulation with IL-15 can induce NK cells to produce IFN-γ and CC chemokines during the acute phase of infection [115]. Also, IL-15 used as a adjuvant to HIV-gag DNA based vaccine enhanced antigen-specific CD8+ T cell proliferation and long term survival of memory CD8+ T cells [116]. In non-human primates, an optimized IL-15 used as an immune adjuvant delivered as DNA induced IFN-γ secretion and control of viral replication [117]. Interestingly, the properties of IL-15 to induce proliferation and function of antigen-specific CD8+ T cells seem

to be independent of CD4+ T cells [118]. However, the most expressive effects observed in immunotherapy with IL-15 was when this cytokine was combined with IL-15Rα. The IL-15/IL-15R complex enhanced by ~50 fold the proliferation activity of IFN-γ producing CD8+ T cells, and ~10 times the protection against tumor challenge [119].

Although this review emphasizes the role of IL-15 and IL-21 mostly in viral immunity, several observations have been made on the use of these two cytokines to prevent or treat tumors [10,120]. IL-21 [121-123] increases anti-tumor NK function and also enhances the magnitude of the CD8 response, acting as the third signal in the differentiation of anti-tumor memory CD8+ T cells, reducing or blocking tumor growth. IL-15 rescues citotoxicity and the proliferation capacity of tumor antigen specific CD8+ T cells, also leading to reduced tumor growth. These results are expected, given the similar nature of the anti tumor and anti-viral responses.

Five-Year View

In order to use cytokines as antiviral therapies or adjuvants in human viral vaccines, specific mechanisms need to be investigated and clarified. A better understanding of secondary effects produced by cytokine treatment is necessary, since some of them are highly novice for humans. Also, the methodology needs to be optimized in order to obtain the most relevant response. The following issues concerning the use of cytokines in viral immunotherapy and vaccines will be a priority in the next years:

(1) Control of lymphocyte proliferation upon cytokine treatment: as was described in this review, IL-15 and IL-21 can both induce CD8+, and some times CD4+, T cell proliferation and survival. The risks of generating an autoimmune disease in this process, especially in susceptible people, are largely unknown.

(2) Bystander proliferation: two controversial hypotheses about the requirements of IL-21 and IL-15 to induce T cell proliferation are currently being investigated by

different groups. One proposes that these cytokines can induce proliferation in the absence of any TCR signaling, especially for memory cells [15,31,71,73,124]. The other argues that antigen is required in this process, especially during the memory phase [81,125,126]. Elucidation of this point is crucial for the design of an efficient protocol of cytokine administration.

(3) Mucosal immunity: several kinds of viruses are transmitted through mucosal surfaces. Research on viral vaccines that induce mucosal immunity, mediated by secretory IgA, must be encouraged. The use of IL-15 and IL-21 for the induction of antiviral mucosal immunity has not been pursued to date.

(4) Other viruses: studies using cytokines to treat or prevent infection by very endemic viruses, especially on less developed countries, are scarce. Dengue virus and Human papillomavirus (HPV) are some examples. Studies similar to the ones performed on HIV, HSV-1 and 2 and hepatitis with IL-15 and IL-21 should be performed for these others viruses.

Expert Comentary

It was anticipated that increased knowledge about the role of cytokines during innate and adaptive viral immunity would translate into effective therapeutic and preventive anti-viral vaccines. However, although there has been much progress on cytokine based anti-viral therapies capable of blocking or reducing viral replication, or yet inducing anti-viral immunity, several viruses still spread around the world and kill millions of people. This may be a reflection of the many mechanisms used by viruses to evade the immune system.

The application of cytokines as adjuvants cannot overcome the natural process of genetic alterations of the virus, but can influence the pattern and quality of the immune response generated, as discussed throughout this review. The efficacy of an antigen specific

immune response is probably established during the priming phase, when cells encounter antigen for the first time. A very interesting study by Prlic and cols has demonstrated that around seven hours of interaction are needed between antigen-presenting DC and CD8+ T cells to ensure significant proliferation in the memory phase [127] Successful memory cell generation begins with early interactions between naive T cell and DC within the inflammatory milieu of the secondary lymphoid organs [128]. IL-15 and IL-21 are part of that inflammatory milieu, and if appropriate levels of these cytokines are provided exogenously, the magnitude of antigen specific CD8+ T cell can be significantly improved. This results from shaping innate immunity through improvement of DC survival, expansion of NK cell number and IFNγ producing cells, and also proliferation of CD4+ T cells to help the virus specific CD8+ T cells. An important observation of use of IL-15 and IL-21 and adjuvants to viral vaccines is that they regulate other cytokines and immunological factors, activating different routes and pathways, leading to a more complete anti-viral response.

Cytokine-based vaccines are currently undergoing clinical trials and the majority of these are now evaluating the safety of application of cytokines in human beings. Most of the studies are directed to development of an HIV-1 vaccine, and they reflect the current epidemiology of this disease, the lack of efficient ways to treat it and the high number of basic studies on mouse and primates models that have been done. Besides safety, doses, routes and combined forms of theses cytokines need to be determined in these clinical trials in order to optimize their results.

KEY ISSUES

- Gamma chain (γc) receptor subunit signaling cytokines play a key role on anti-viral immunity.

- IL-15 induces innate immunity against viruses through control of IFNα/β production, NK cell proliferation and DC activation potential.
- IL-15 controls the number of antigen specific CD8+ T cell that die or become memory cells, and these are highly dependent on IL-15 for homeostatic proliferation.
- IL-21 can be an additional signal provided by CD4+ T cells to fully activate CD8+ T cells upon infection.
- During viral activation after latency, IL-21 rescues the expression of surface molecules CD27 and CD28 on effector lymphocytes, these molecules reflecting proliferation and activation, respectively.
- Efficient antiviral vaccines and therapies are designed utilizing IL-15 and IL-21 in combination, since these two cytokines together have a profound impact on memory CD8+ T cell proliferation and function.
- IL-15Rα can potentiate the effect of IL-15 as an adjuvant to viral vaccines by around fifty times. This is related to transpresentation and bioavailability of the IL-15/IL-15Rα complex.

Figures

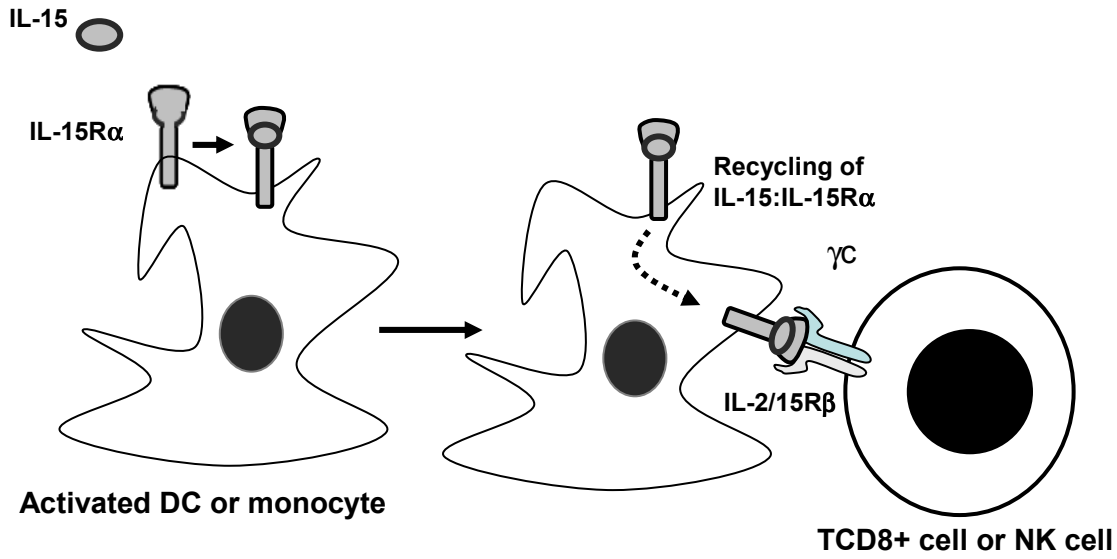

Figure1: Recycling and transpresentation of IL-15:IL15Rα. During an infection IL-15 production is increased in surrounding area, as a result of macrophages or DC activation . This cells also up-regulate the expression of IL-15Rα. IL-15 binds to its receptor on surface of the cell and the complex is internalized. The complex is recycled and transpresented to a CD8+ T cell or NK cell that do express IL-2/15Rβ and γc. (Adapted from Schluns, 2003, Nat Reviews Immunology)

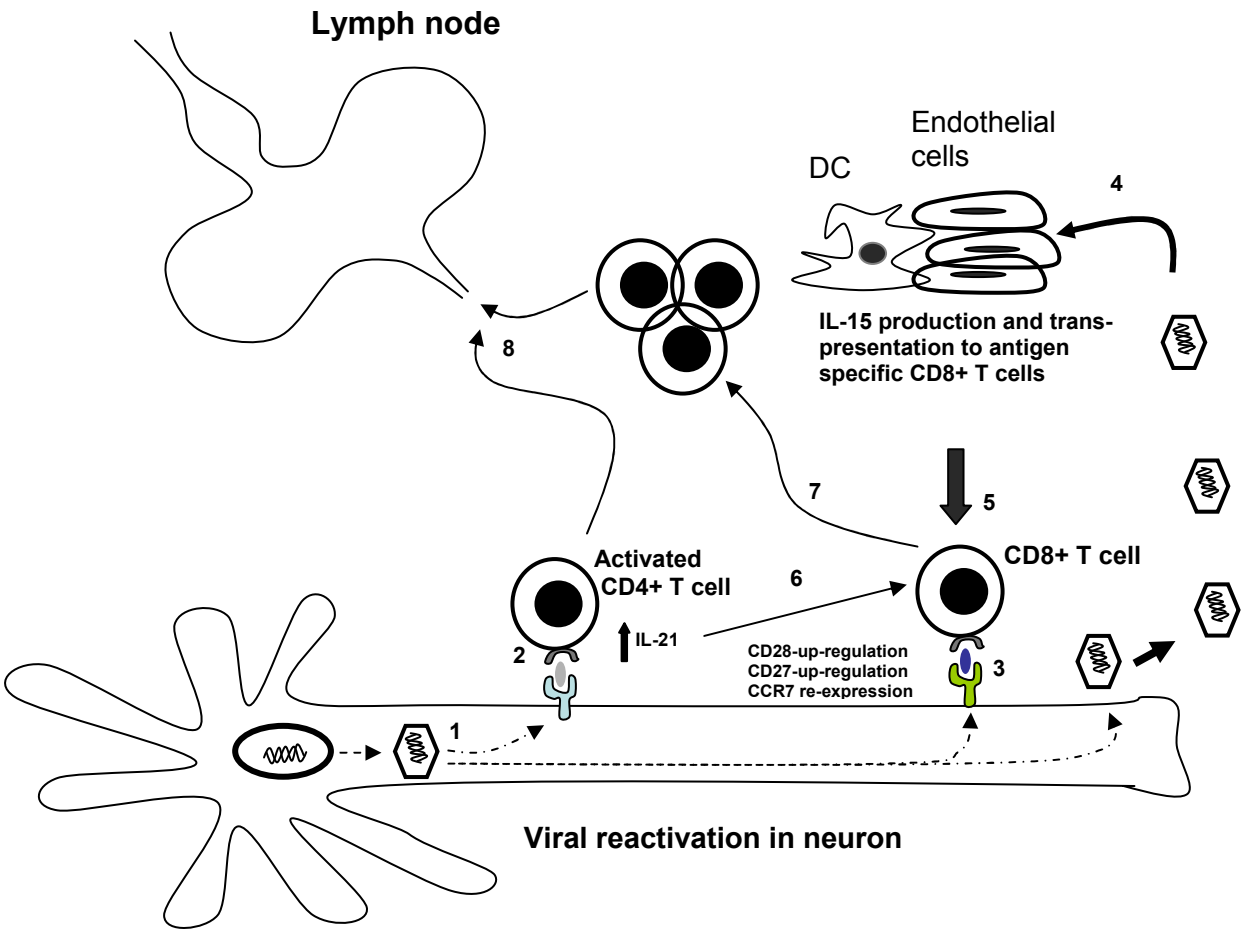

Figure 2: Mechanism proposed for antiviral IL-15 and IL-21 interaction during viral reactivation of HSV-1 from latency. Upon HSV reactivation, IL-15 and IL-21 can act synergistically to improve the magnitude and function of antigen-specific effector memory CD8+ T cell. 1) Viral reactivation inside the neuron; 2) Presentation to antigen-specific CD4+ T cell; 3) Presentation to antigen-specific CD8+ T cell; 4) Virus released from infected neuron activates DC and epithelial to cell to produce IL-15 and IL-15R; 5)The complex IL-15:IL-15Rα is trans-presented to effector memory CD8+ T cells that express IL-2/15Rβ and γc; 6) IL-21 produced by activated CD4+ T cell induce up-regulation of CD27and CD28 and re-expression of CCR7 on CD8+ T cells; 8) Effector memory CD8+ migrate to local lymph node and proliferate.

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5 - Considerações Finais

Ainda não existe cura para a grande maioria das doenças virais, sendo que muitas delas matam milhões de pessoas a cada ano. Além disso, em algumas infecções virais, a inserção do DNA viral no genoma humano, cria um estado semelhante a um "transgênico", no qual o homem fica portador do gene viral por toda a vida. De acordo com o padrão de expressão gênica, podemos classificar dois tipos de infecção viral, a crônica e a latente. Na infecção latente, o gene é expresso ou não, dependendo, principalmente, da resposta imunológica. A expressão desses genes com manifestação clínica chama-se reativação viral, bastante comum na infecção pelos alfa-herpesvírus, no caso o HSV-1. A quantidade, a função e o momento de atuação dessas células na imunidade antiviral é determinada pela ação das citocinas, os principais mediadores imunológicos. Nesse trabalho foram utilizadas duas citocinas determinantes na biologia de linfócitos T $CD8^+$ de memória, a IL-15 e a IL-21, para aprimorar a resposta dessas células em um modelo de infecção herpética. Vários aspectos foram analisados, desde a construção das citocinas recombinantes até o estudo da resposta de memória *in vivo*. Alguns pontos observados no trabalho foram mais relevantes e contribuem bastante para a o desenvolvimento de vacinas anti-virais:

5.1 - Otimização da expressão da IL-15 in vitro: A IL-15 é uma citocina expressa por uma variedade de células e apresenta efeitos estimulatórios sobre linfócitos T $CD4^+$, linfócitos T CD8+ e células NK (SANDAU et al, 2007; GILL et al, 2005). O mecanismo de expressão dela é extremamente regulado por *splicing* alternativo, a partir do qual duas isoformas são produzidas, uma com o peptídeo sinal longo (L/IL-15) e outra como peptídeo sinal curto (S/IL-15) (NISHIMURA et al, 2000). Embora a forma L/IL-15 seja mais direcionada para a secreção, é muito difícil sua detecção no sobrenadante de células transfectadas com plasmídeo que codifica para o gene da IL-15. Isso é resultado, em parte, da baixa expressão e também da apresentação cruzada, discutida no capítulo 3, que faz com que a IL-15 fique localizada na superfície da célula. Uma forma otimizada da IL-15 foi elaborada trocando a seqüência sinal da IL-15 pela seqüência sinal da IL-2, que dirige um padrão se secreção normal. Essa troca foi realizada por estratégia de amplificação com oligonucleotídeos iniciadores com a seqüência completa do gene da seqüência sinal da IL-15. A partir dessa substituição, foi observado um aumento da secreção da IL-15 *in vitro* na ordem de oito vezes mais, comparado com a IL-15

não modificada (anexo 1). Essa construção gênica com IL-15 está sendo utilizada para estudos de estimulação de células in vitro para terapia celular.

5.2 - Aplicação do promotor de ferritina na expressão de citocinas in vitro e in vivo: As citocinas são bons adjuvantes para o desenvolvimento de terapias e vacinas, os resultados apresentados nesse trabalho ajudam a comprovar isso. Entretanto, elas apresentam um custo elevado, se obtidas comercialmente, e alternativas para obtenção das citocinas de forma recombinante devem ser estimuladas. Nesse trabalho foi utilizado o vetor de expressão pVIVO-2, que apresenta um sistema de expressão controlado por dois promotores que funcionam de forma independente um do outro, tanto *in vivo* quanto *in vitro*. Esse promotor é controlado em condições normais pelos níveis de ferro do organismo, mas nesse sistema a dependência do ferro foi removida através da construção de um mutante constitutivo (MANUAL INVIVOGEN, 2008). O promotor de citomegalovírus é um dos mais utilizados na imunização genética, mas os níveis de expressão obtidos ainda não são os ideais, pois esquemas de imunização com quantidades altas de plasmídeos e vários reforços devem ser aplicados. Foi realizado um estudo comparativo entre o promotor e ferritina e o citomegalovírus, e os resultados demonstraram que o promotor de ferritina é superior. Nos estudos com IL-15, a troca da seqüência sinal e a utilização do promotor de ferritina aumentaram em 100 vezes a expressão, comprado com a IL-15 normal, sob o controle de promotor de citomegalovírus (anexo 1). Outra característica importante avaliada nesse trabalho, foi a expressão in vivo da IL-21 sob controle do promotor de ferritina, quantidades aproximadas de 500pg/mL de IL-21 foram obtidas no músculo de animais tratados com o plasmídeo (figura 1C, capítulo 2). Esse dado torna possível uma comparação entre a quantidade de citocina no organismo e a resposta imune obtida posteriormente. Não foi possível a detecção da IL-15 *in vivo*, pois os sistemas disponíveis para esse tipo de análise dessa citocina são pouco sensíveis.

5.3 - Proliferação de células T CD8+ *in vitro*: As células T $CD8^+$ proliferam como resultado da sinalização de IL-15 e IL-21 (KUTZLER et al, 2005; ZENG et al, 2005). Embora um experimento específico de análise da proliferação não tenha sido aplicado, os resultados do cultivo de esplenóctios de camundongo com a IL-21 produzida mostraram que essa citocina aumenta a freqüência das células T $CD8^+$ (Figura 2, capítulo 2). Na verdade, os resultados sugerem que a IL-21 ajuda na sobrevivência de células T $CD8^+$ in vitro, o que está de acordo com resultados de outros grupos (OSTIGUY et al, 2007). Embora os efeitos tenham sido

observados tanto na presença quanto na ausência ativação das células T, na ativação os resultados foram melhores. Esse resultados indicam que a IL-21 produzida a partir do plasmídeos pVIVO-2 tem atividade positiva sobre as células T $CD8^+$, podendo ser utilizada na expansão in vitro. Outro fenômeno observado, mas pouco discutido na discussão do capítulo 2, foi em relação a redução do número de células $CD3^-$ observado na figura 2 do capítulo 2. Isso pode ser resultado da morte de células B, nas quais a IL-21 induz apoptose sob estimulação inespecífica (MEHTA et al, 2003), ou células NK, que também diminuem o potencial proliferativo como resultado da ação da IL-21 (BRADY et al, 2004; KASAIAN et al, 2002).

5.4 - Aumento da resposta primária CD8+ efetora: A administração de pIL-15 ou pIL-21 previamente a infecção herpética levou a um aumento da quantidade de células T $CD8^+$ antígeno específicas e secretoras de IFN-γ (Figura 4, capítulo 2). Esse resultado indica que as citocinas administradas na forma de DNA podem ser utilizadas para profilaxia e terapia do HSV-1. O momento de análise foi no dia 12 após a infecção, fase na qual as células T $CD8^+$ estão entrando na fase de declínio. É possível que a IL-15 resgate células T $CD8^+$ efetoras da morte. Em concordância com os resultados obtidos *in vitro*, o tratamento com IL-21 aumentou também a quantidade de células T CD8+. Os números de células obtidos para pIL-15 e pIL-21 não são elevados, quando comparados com animais que receberam somente o vetor. Entretanto, deve-se considerar que os animais foram tratados com vírus e o limiar de resposta atingida pelo vetor fica também alto. Portanto, administração da IL-15 e IL-21 na forma de DNA é capaz de aumentar a resposta primária de $CD8^+$ na infecção ao HSV-1.

5.5 - Expansão clonal: Nesse trabalho foi realizado um estudo de determinação das quantidade de células SSIEFARL transgênicas necessárias para a transferência celular, e capazes de apresentar uma proliferação mais eficiente após infecção com o antígeno. Os resultados observados mostraram que quanto menor o número de células transgênicas transferidas, maior é a razão de divisão dessas células no animal receptor (Figura 5, capítulo 2). Em termos de terapia celular, isso significa que a transferência de baixos números de células de um mesmo clone proporciona uma menor competição pelo mesmo antígeno, apresentado pelas DCs. Por outro lado, em termos de resposta imunológica, isso significa que mais células antígeno-específicas são produzidas durante a infecção no animal receptor, quando baixas quantidades são transferidas. Essas células podem ter mais chances de se

tornarem células de memória eficientes, pois elas são resultantes da proliferação após encontro com antígeno no animal hospedeiro (HATAYE et al, 2006).

5.6 - Efeito de pIL-15 e de pIL-21 na resposta CD8+ de memória ao HSV-1: A proliferação e a função de linfócitos de memória é dependente de diferentes fatores, antígenos, citocinas e expressão de moléculas de adesão (HARTY et al, 2008). Entretanto, ainda não se conhece o nível de dependência das células T $CD8^+$ a cada um desses estímulos, principalmente durante a resposta de memória. Nesse estudo foi analisado o efeito proliferativo da IL-15 e da IL-21 nas células T CD8+ de memória, quando injetadas sozinhas os com antígeno durante a fase de memória. Os resultados indicam que a administração do DNA das citocinas aumentaram a quantidade das células T $CD8^+$ transferidas, quando os plasmídeos das citocinas foram administrados sozinhos, combinados ou com antígeno (Figura 6, capítulo2). Esses resultados sugerem que as citocinas podem dirigir um proliferação homeostática de células T $CD8^+$, em células de memória no baço. Nesse experimento era esperado uma resposta mais potente no grupo de animais tratados com a combinação de pIL-15 e pIL-21, como observado nos estudos de Zheng e cols, entretanto, isso não foi observado (ZENG et al, 2005). O modelo utilizado para o estudo foi de animais normais não *knockout* para os genes da IL-15 ou IL-21, é possível que a resposta obtida com cada plasmídeo tenha a contribuição dos efeitos da citocina endógena. Um estudo mais específico pode ser conduzido utilizando modelos de animais *knockout* para IL-15 e IL-21. Resultados na mesma magnitude foram observados nas células com fenótipo de memória ($CD44^{high}$). Além disso, a associação de antígeno também não aumentou muito a resposta, quando comparado com a citocina sozinha. Isso também pode estar relacionado com o fato do herpes produzir uma infecção latente e o antígeno estar presente em algum momento (SHERIDAN et al, 2007), o que tornaria a administração de mais antígeno desnecessária ou excessiva. Embora não seja estatisticamente diferente dos tratamentos únicos, quando foi combinado o pIL-15, pIL-21 e pgB, a resposta obtida nesse grupo foi a que mais diferiu dos animais que receberam somente o vetor, em todas as análises ($CD8^+$, $CD44^{high}$ Tet^+ e $CD8^+IFN\gamma$). Esse resultado sugere que quando se promove mais sinais para as células T $CD8^+$ de memória na infecção herpética, elas proliferam mais e são capazes de fazer uma resposta efetora mediada por IFN-γ. Entretanto, a melhor forma de provar que os efeitos observados são determinantes na resposta ao HSV-1 *in vivo* seria um estudo com desafio com o vírus na fase de memória, após o tratamento.

Mecanisticamente, a IL-15 é capaz de aumentar a proliferação de células T $CD8^+$ na ausência de estimulação via TCR (GUIMOND et al, 2005). Por outro lado, essas células

reduzem a expressão do marcador CD28, importante para a ativação celular, e também do marcador CD27, importante para a proliferação celular. A IL-21 tem a capacidade de prevenir a perda de CD28 e resgatar ou reduzir a expressão de CD27 e CCR7 (ALVES et al, 2005; GAMADIA, a t al, 2004). A combinação dessas duas citocinas promove sinais para as células T $CD8^+$ antígeno-específicas proliferarem e se tornarem ativadas. Um esquema desse sistema pode ser observado na figura 2.

A utilização de pIL-15 ou pIL-21 durante a fase de memória não foi testada antes, a maioria dos estudos aplica as citocinas na fase de indução ou na fase efetora e analisa a resposta de memória. Os dados apresentados nesse trabalho contribuíram para demonstrar que a utilização do DNA dessas citocinas na fase de memória induz a proliferação homeostática de células T CD8+ de memória ao HSV-1, e que a administração de antígeno não é determinante, mas melhora a resposta, no caso dessa infecção latente.

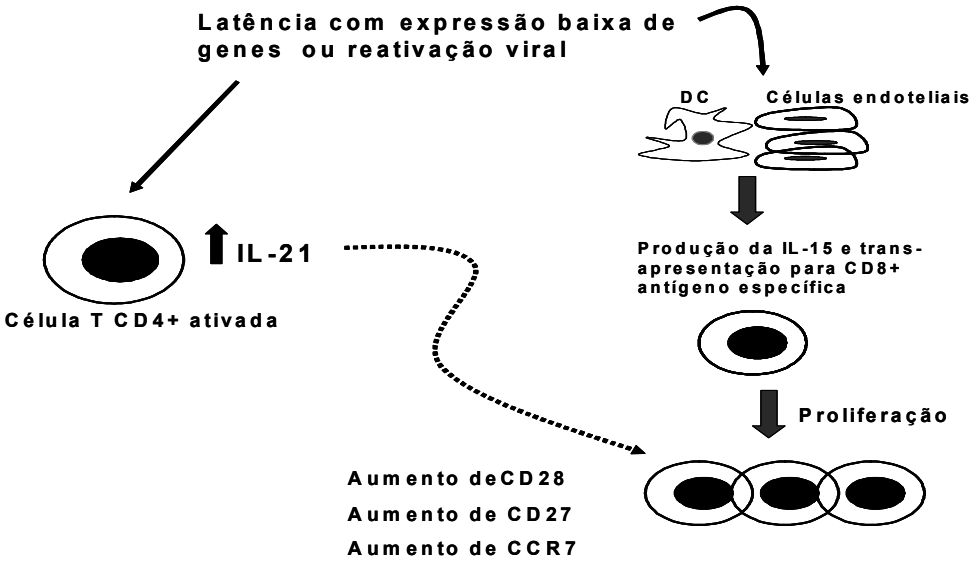

Figura 2: Modelo proposto para o efeito sinergistico da IL-15 e da IL-21 na magnitude das células T CD8+ durante a infecção viral latente ou reativação. Durante a reativação viral um ambiente inflamatório é gerado e as DC e células endoteliais produzem IL-15, que é trans-apresesntado para as CD8+ naive na forma de um complexo com IL-15R, induzindo a proliferação e perda de CD28, CD27. As células T CD4+ produzem IL-21 como resultado da reativação viral. A IL-21 previne a perda de CD28 nas células CD8+ naive e resgata a expressão de CD-27 e CCR7 nas células T CD8+ effetoras ou de memória.

6 – Conclusão

A citocina IL-21 quando utilizada na forma de terapia genética pode ser detectada diretamente no tecido local de injeção, sendo essa detecção relacionada com o nível de expressão do plasmídeos que codifica a citocina *in vivo*. O promotor eucariótico utilizado é determinante para a obtenção dos nívies satisfatórios de expressão dos plasmídeos *in vitro* e *in vivo*. Entretando, mesmo com modificação molecular e utilização do promotor de ferritina, mais eficiente nos estudos conduzidos, a expressão da IL-15 *in vivo* não foi detectada, mas isso é relacionado com os sistema de detecção e biologia da citocina, e não com a expressão. *In vitro*, ambas as citocinas clonadas apresentaram resultados positivos sobre a freqüência de células T $CD8^+$, a IL-21 diretamente sobre esplenóctios e a IL-15 sobre uma linhagem de linfócitos T citotóxicos murinos. A estimulação de TCR, que equivale à adição de antígeno, aumentou a resposta, mas não é determinante para a ação da IL-21 nas células T $CD8^+$ *in vitro.*

No modelo de infeção herpética os plasmídeos pIL-15 e pIL-21 aumentaram a quantidade de células T $CD8^+$ pordutoras de IFN-γ na resposta primária. Os plasmídeos funcionaram como adjuvantes para indução de uma resposta específica. Na fase de memória da infeção herpética latente, os plasmídeos das citocinas aumentaram os números de células $TCD8^+$ ao vírus da herpes, essas células também são produtoras de IFN-γ, pois as células duplo positivas $CD8^+IFN\text{-}\gamma^+$ aumentam comparativamente aquelas que são somente $CD8^+$. A combinação de pIL-15, pIL-21 e pgB apresentou as maiores magnitudes de resposta dos marcadores celulares analisados, entretanto, qualquer uma delas quando utilizada sozinha, foi melhor que o vetor.

Esses resultados indicaram que no caso de uma vacina ou terapapia para HSV-1, a aplicação dos plasmídeos que codificam citocinas na fase de memória pode aumentar os reservatórios de células T $CD8^+$. É prpvável que mecanismos de proliferação homeostática estão envolvidos nesse processo.

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