ATENUAÇÃO DO VÍRUS VACINAL DO SARAMPO : INFECÇÃO SUB-ÓTIMA DO TECIDO LINFÁTICO E ALTERAÇÃO DO TROPISMO

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SUMÁRIO

PARTE V – ARTIGO

PARTE VI – ANEXOS

LISTA DE ABREVIATURAS E ACRÔNIMOS

AC:	Antes de Cristo
AIDS:	Síndrome da Imunodeficiência Adquirida / *Acquired Immunodeficiency Syndrome*
APC:	Aloficocianina / *Allophycocyanin*
CA:	California
CCR5:	Receptor da quimiocina CC – 5 / *CC Chemokine Receptor 5*
CD:	"Agrupamento de Diferenciação" / *Cluster of Differentiation*
CDK:	Quinase dependente de ciclina / *Cyclin Dependent Kinase*
CXCR4:	Receptor da quimiocina CXC – 4 / *CXC Chemokine Receptor 4*
DC:	Célula dendrítica / *Dendritic Cell*
DC-SIGN:	Não integrina capturadora de ICAM3 específica das células dendríticas / *Dendritic Cell Specific ICAM3 Grabbing Non-integrin*
DMEM:	Meio de Eagle modificado por Dulbecco/ *Dulbecco's Modified Eagle's Medium*
DNA:	Ácido Desoxirrubonucleico
EDA:	Encefalomielite Disseminada Aguda
FCS:	Soro Fetal Bovino / *Fetal Calf Serum*
FITC:	Isotiocianato de fluoresceína / *Fluorescein Isothiocyanate*
HIV:	Vírus da imunodeficiência humana / *Human Immunodeficiency Virus*
HLA:	Antígeno leucocitário humano / *Human Leukocyte Antigen*
ICAM3:	Molécula de adesão intracellular 3/Intracellular Adhesion Molecule 3
IFN:	Interferon

Ig:	Imunoglobulina
IgA:	Imunoglobulina isotipo A
IgE:	Imunoglobulina isotipo E
IgG:	Imunoglobulina isotipo G
IgM:	Imunoglobulina isotipo M
IL:	Interleucina
IPO:	Instituto de problemas de oncologia, Kiev / *Institute of Problems of Oncology, Kiev*
MCP:	Proteína Cofatora de Membrana / *Membrane Cofactor Protein*
MHC:	Complexo maior de histocompatibilidade / *Major Histocompatibility Complex*
MMR:	Vacina contra sarampo, cachumba e rubéola / *Measled Mumps Rubeola*
MV:	*Measles Virus*
MOI:	Multiplicidade de infecção / *Multiplicity Of Infection*
NM:	Nanômetros
NICHD:	Instituto nacional de saúde da cirança e do desenvolvimento humano *National Institute of Child Health and Human Development*
NIH:	Institutos nacionais de saúde / *National Institutes of Health*
NK:	*Natural Killer*
NKT:	*Natural Killer T*
NR:	Receptor da proteína N (nucleoproteína)
OMS:	Organização Mundial da Saúde

PCR:	Reação em Cadeia da Polymerase
	Polymerase Chain Reaction
PE:	Ficoeritrina / *Phycoerythrin*
PES:	Panencefalite Esclerosante Subaguda
PIB:	Produto Interno Bruto
PROTEÍNA F:	Fusão / *Fusion*
PROTEÍNA H:	Hemaglutinina / *Hemagglutinin*
PROTEÍNA L:	Maior / *Large*
PROTEÍNA M:	Matriz / Membrana */ Matrix / Membrane*
PROTEÍNA N:	Nucleoproteína / *Nucleoprotein*
PROTEÍNA P:	Fosfoproteína / *Phosphoprotein*
RANTES:	*Regulated on Activation, Normal T cell Expressed and Secreted*
RNA:	Ácido Ribonucleico
RT-PCR:	Reação em Cadeia da Polimerase com Transcriptase Reversa
	Reverse Transcriptase Polymerase Chain Reaction
SD:	Desvio Padrão / *Standard Deviation*
SEM:	Erro Padrão / *Standard Error of the Mean*
SLAM:	Molécula de Ativação Linfocítica de Sinalização /
	Signaling Lymphocyte Activation Molecule
SNC:	Sistema Nervoso Central
STAT:	Transdutor de Sinal e Ativador de Transcrição /
	Signal Transducer and Activator of Transcription
TBC:	Tuberculose

TCID:	Dose Infecciosa para Cultura de Tecidos
	Tissue Culture Infective Dose
TH:	T auxiliar / *T Helper*
TLR:	Receptor "*Toll Like*"
TNF:	Fator de Necrose Tumoral / *Tumor Necrosis Factor*
TRAIL:	Ligante Indutor de Apoptose Relacionado ao TNF
	TNF Related Apoptosis Inducing Ligand
TRECs:	Círculos de Excisão do Receptor da Célula T
	T cell Receptor Excision Circles
US:	Estados Unidos / *United States*
UV:	Ultra Violeta
VAC:	Vacinal
VERO:	*Verda Reno*
VERO/hSLAM:	Células VERO que expressam SLAM humano
Vs:	Versus
WT:	Selvagem (linhagem) / *Wild Type*

LISTA DE FIGURAS

RESUMO

Condack, C.E. Atenuação do vírus vacinal do sarampo: infecção sub-ótima do tecido linfático e alteração do tropismo. Belo Horizonte: UFMG. 85p. Tese de Doutorado em Ciências da Saúde (área de concentração: Saúde da Criança e do Adolescente).

Os mecanismos de atenuação do vírus vacinal do sarampo ainda não foram suficientemente caracterizados. Uma vez que a linhagem vacinal consegue penetrar as células através do receptor CD46 além do receptor primário do vírus do sarampo CD150 / SLAM (molécula de ativação e de sinalização linfocitária) nós nos perguntamos se (e como) o seu tropismo está alterado. Em tecido amigdaliano humano, a linhagem vacinal infecta linfócitos T virgens (CD45RA+ CD62L+), que expressam SLAM em pequena quantidade, com muito mais eficiência que as linhagens selvagens. Por outro lado, a linhagem vacinal infecta significativamente menos linfócitos B, macrófagos e células NK que as linhagens selvagens. Os níveis de infecção das linhagens selvagens se correlacionam com a freqüência de expressão da SLAM, sendo mais elevados nos linfócitos B, que apresentam níveis de infecção entre 40-55%. As células T que expressam SLAM são mais facilmente infectáveis que as células B que expressam essa molécula. Desta forma, a atenuação da linhagem vacinal parece ser causada por uma alteração no seu tropismo, juntamente com uma replicação menos eficiente.

ABSTRACT

Condack, C.E Measles virus vaccine attenuation: suboptimal infection of lymphatic tissue and tropism alteration. Belo Horizonte: UFMG, 85p. PHD Thesis in Health Sciences (domain: Child and Adolescent Health).

The mechanisms of measles virus (MV) vaccine attenuation are insufficiently characterized. Because the vaccine strain can enter cells through CD46 in addition to the primary MV receptor signaling lymphocyte activation molecule (SLAM, CD150), we asked whether and how its tropism is altered. In human tonsillar tissue, this vaccine strain infects naive (CD45RA+ CD62L+) T lymphocytes, which express SLAM very infrequently, with much higher efficiency than do wild-type strains. By contrast, it infects B-lymphocytes, macrophages, and NK cells with significantly lower efficiencies than those of wild-type strains. Infection levels by wild-type strains correlate with the frequency of SLAM expression, and are highest in B-cells, which are 40-55% infected. SLAM-expressing T-cells are more readily infected by all MV strains than are SLAM-expressing B-cells. Thus, vaccine attenuation may be caused by tropism alteration in combination with suboptimal replication.

PARTE - I

REVISÃO DA LITERATURA

1. INTRODUÇÃO

1.1 Dados epidemiológicos

O sarampo é uma doença relativamente nova em humanos, com provável origem na antiga suméria por volta de 3000 AC. Seu vírus pertence à família Paramyxoviridae e se assemelha filogeneticamente ao morbilivírus animal causador da peste bovina [1]. Ainda hoje, o sarampo é categorizado como uma das doenças infecciosas mais freqüentes e mortais, sendo a entidade clínica mais importante passível de prevenção por vacina [2]. Apesar de vacinas altamente eficazes contra o sarampo terem sido desenvolvidas há décadas [3], ainda hoje podemos observar altas taxas de incidência e de mortalidade em crianças, principalmente em países com baixo PIB per capta [4].

Em termos mundiais, temos presenciado nos últimos anos uma diminuição progressiva no número de infectados e de óbitos decorrentes do sarampo. Em 2006, a Organização Mundial da Saúde estimou 242.000 óbitos, na sua maioria crianças [4], contra uma mortalidade estimada em 777.000 óbitos no ano 2000 [5]. A mortalidade infantil se mostra especialmente desafiadora quando se leva em conta o grande número de casos em lactentes menores de um ano [6]. Nessas crianças, especialmente nas menores de nove meses, observa-se uma janela de tempo durante a qual os anticorpos maternos impedem uma resposta vacinal eficaz, mas não previnem completamente contra a infecção pelo vírus selvagem [7]. Em alguns países em desenvolvimento uma criança em cada três chega a se infectar nos primeiros meses de vida [8].

Com o aumento da cobertura vacinal, foi notada uma diminuição particularmente significativa na incidência de sarampo nos países africanos, que passaram da primeira posição para a vice-liderança no número total de óbitos; a primeira posição está ocupada atualmente pelos países do sudeste asiático [4]. Apesar da diminuição na incidência, a mortalidade continua alta em muitos países em desenvolvimento, principalmente em crianças desnutridas [9] e naquelas cujos pais apresentavam baixa escolaridade [6].

Apesar do sarampo estar controlado no Brasil e nos Estados Unidos, há mais de uma década surtos esporádicos continuam a existir, como o ocorrido em Indiana (US) em 2005 [10], ou como o ocorrido no início de 2007, no estado da Bahia [11]. Tais surtos geralmente decorrem do contato de pessoas susceptíveis com indivíduos infectados provenientes de áreas onde ainda não existe controle adequado da doença, uma vez que o

vírus pode ser transmitido mesmo na fase prodrômica da doença (antes do surgimento do *rash* cutâneo) [12]. O insucesso no controle da transmissão ocorre, portanto, devido não somente à vigilância inadequada, com falhas na cobertura vacinal, mas também devido ao uso de vacinas com qualidade deficiente, pois a vacina com o vírus atenuado perde rapidamente a sua infectividade, uma vez reconstituída. Ainda, o vírus atenuado é inativado pela luz e pelo calor, perdendo metade de sua potência se mantido a 20°C e toda a sua potência em 1 hora a 37°C [13].

A periodicidade com que esses surtos ocorrem depende, desta forma, do número de susceptíveis de determinada população [14]; sendo que a transmissão é possível mesmo em populações com menos de 7% de indivíduos soronegativos [15]. Estima-se que 90% dos indivíduos não vacinados expostos acabariam se infectando [16].

1.2 O vírus e as proteínas codificadas pelo seu genoma

O vírus do sarampo foi inicialmente isolado por Enders em 1954 [17], sendo um membro da família Paramyxoviridae, gênero morbilivírus [16]. Os vírions são pleomórficos, com tamanho variando de 100 a 300nm [1] e genoma de aproximadamente 15-16 quilobases [15,18]. O genoma viral é composto de uma fita simples de RNA de polaridade negativa [16] que codifica 8 proteínas – N, P, C, V, M, F, H e L [1, 16] (Fig.1).

As proteínas H (hemaglutinina) e F (fusão) estão localizadas no envelope viral, mediando a ligação do vírus e sua entrada nas células permissivas [1,19]. Essas proteínas são também expressas na superfície de células infectadas [16]. A ligação inicial do vírus com a superfície das células é feita pela proteína H, enquanto a sua entrada é mediada pela interação da proteína F com a membrana celular [20](Fig.1). A proteína H também estaria envolvida na determinação do tropismo viral [21], sendo que a proteína do vírus selvagem (e não a do vacinal) seria capaz de ativar células via TLR-2 (receptores *toll-like* 2), contribuindo para a ativação imune e para a disseminação viral pelo aumento na expressão do seu receptor – CD150 – em monócitos [22].

Os receptores *toll-like* funcionam como "reconhecedores de padrão", sendo importantes na identificação de componentes microbianos "estranhos" ao hospedeiro. Sua ativação desencadeia respostas imunes que visam à destruição dos organismos invasores

[23]. O vírus do sarampo, por outro lado, parece induzir a ativação do TLR-2 em beneficio próprio.

De forma interessante, foi descrito que a substituição do aminoácido tirosina pelo aminoácido asparagina na posição 481 da hemaglutinina viral seria responsável não somente pela perda da capacidade to vírus selvagem em estimular os TLR-2, mas também seria capaz de levar as linhagens selvagens a penetrarem nas células usando o receptor CD46 [22] (característica marcante das linhagens vacinais em relação às selvagens, que estaria associada à atenuação das linhagens vacinais) [24].

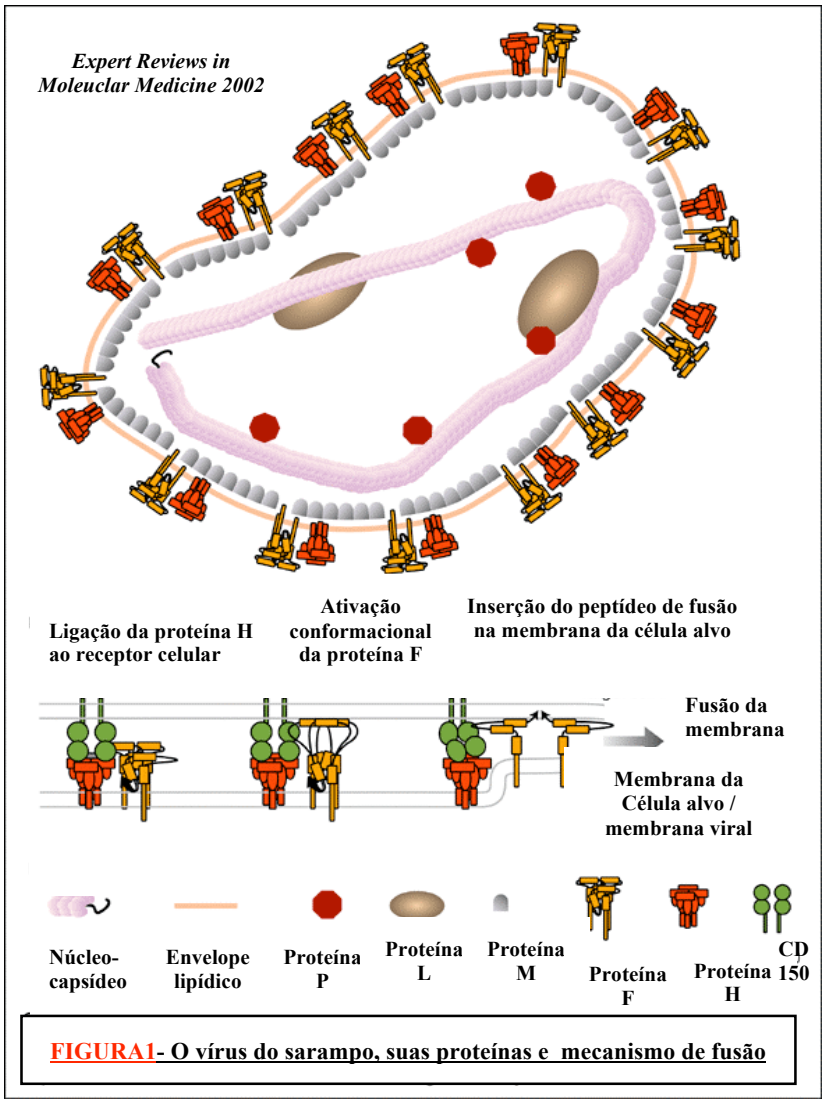

FIGURA1- O vírus do sarampo, suas proteínas e mecanismo de fusão

A terceira proteína associada ao envelope é a M (matriz), que fornece uma interface entre o envelope e o cerne viral, composto pelo RNA viral juntamente com a proteína do nucleocapsídeo (N), a fosfoproteína (P) e a proteína maior (L) [25].

A proteína M teria um papel na montagem do vírion e no processo de "brotamento" viral na superfície celular [1].

A proteína N é a mais abundante de todas as proteínas virais e possui papel primordial na replicação do RNA [1]. Ela parece envolver e proteger o ácido nucléico genômico, formando um complexo de replicação juntamente com as proteínas L e P [25].

A proteína maior (L) é uma polimerase de RNA dependente de RNA responsável pela síntese do antigenoma viral [16].

A proteína P, por sua vez, é codificada pelo gene P (que também codifica as proteínas não estruturais C e V) [19]. Sua função é ajudar na montagem da ribonucleoproteína, regulando a transcrição e a replicação viral [16].

Apesar de não essenciais *in vitro*, parece haver um papel importante para as proteínas C e V na patogênese *in vivo*. Takeuchi e cols mostraram que a proteína C preveniria a morte celular e seria necessária para a infecção persistente pelo vírus do sarampo [26]. Experimento com ratos transgênicos que expressavam o receptor CD46 também mostrou menor patogenicidade das linhagens virais que não apresentavam as proteínas C e V [27].

O vírus do sarampo é sorologicamente monotípico, mas a caracterização genética das linhagens selvagens identificou oito clades (A-H) que foram divididas em 22 genótipos e um genótipo proposto. No Brasil, os genótipos detectados foram A, C2, D5 e D6 [28]. Apesar da variabilidade genotípica detectada, não foram notadas diferenças biológicas entre os diferentes genótipos [28], tais como diferenças na apresentação clínica da doença [2, 28,29], possibilidade de desenvolver seqüelas graves ou variabilidade na sensibilidade do diagnóstico laboratorial [28]. A ausência de diferenças biológicas entre os genótipos talvez advenha do fato da classificação genotípica ser determinada com base em diferenças nas proteínas N e H [28], não levando em consideração as diferenças nas sequências das demais.

As linhagens vacinais, por outro lado, apresentavam menor variabilidade genética, pertencendo apenas ao genótipo A.

Se por um lado sabemos que a variabilidade genética entre as linhagens selvagens não é capaz de levar à grande variabilidade biológica, por outro se torna intrigante como as linhagens vacinais se diferenciariam das selvagens, de forma a não causarem doença sendo, por outro lado, capazes de induzir resposta imune duradoura. Estudos realizados com este fim mostraram que poucas eram as mudanças nas seqüências de nucleotídeos entre essas linhagens, com apenas pequenas alterações nos aminoácidos codificados, sendo as mais significativas aquelas notadas na codificação das polimerases (proteínas L e P) [18,30] e / ou nas proteínas acessórias (V e C) [18] que levavam à diminuição da capacidade de replicação e da capacidade de indução de sincício. Mutações na proteína M também foram descritas [30]. Diferenças nas codificações das proteínas P, V, C e M também foram notadas quando feita a comparação do genótipo de duas linhagens isoladas em diferentes tipos celulares, mas derivadas de apenas um paciente, isolamento esse capaz de levar a linhagem inicialmente patogênica a não mais ser capaz de induzir doença em macacos [31].

1.3 Receptores virais

Em 1993 foi identificada a primeira molécula de superfície com capacidade de se ligar ao vírus do sarampo, funcionando como receptor viral - CD46 [32,33]. Além do vírus do sarampo, o CD46 também serviria como receptor para outro vírus de RNA (vírus da diarréia viral bovina), dois vírus de DNA (o vírus do herpes humano tipo 6 e o adenovírus) e duas bactérias (*Streptococcus pyogenes* e *Neisseria*) [34]. O CD46, também conhecido como MCP (*membrane cofactor protein*), é uma proteína de superfície que interage com as frações C3b e C4b do complemento, inibindo a progressão da cascata de ativação, evitando a lise celular. Sua distribuição é ampla, sendo encontrado em leucócitos, células epiteliais e células endoteliais [35].

Estudos posteriores mostraram, entretanto, que a maioria das linhagens selvagens do vírus do sarampo não usaria o CD46 como receptor [36, revisto em37]. Apesar de algumas linhagens selvagens serem capazes de se ligar ao CD46 [38], o seu principal receptor seria o CD150 (SLAM / IPO3) [39]. O CD150 seria também o principal receptor para outros morbilivírus que infectam animais, como o vírus da cinomose canina e o vírus da peste bovina [40]. Considerando as linhagens vacinais / atenuadas, admite-se atualmente que a já referida mutação em um aminoácido na posição 481 da proteína hemaglutinina (H) [24]

favoreceu a sua capacidade de se ligar e penetrar nas células via CD46, mantendo, por outro lado, o uso do CD150 como receptor [36].

Há muito se sabia que a ligação da proteína H do vírus do sarampo com o CD46 resultava na internalização [41,42] deste receptor e hoje acredita-se que esta internalização poderia ser um dos fatores responsáveis pela atenuação viral, pois levaria à maior lise das células infectadas (pela ação do complemento) com menor disseminação viral [7].

O CD150 (SLAM – *signaling lymphocyte activation molecule* / IPO-3) foi inicialmente descrito como uma molécula de superfície encontrada em linfócitos B ativados, em células T de memória (CD45RO+ CD45RA-) e em timócitos CD4+ CD8+ CD45RO+ CD45RA- [43]. Posteriormente, notou-se que também seria encontrado em células dendríticas ativadas [44], células T CD56+ [45], linfócitos T e B ativados e timócitos imaturos [45,46]. A sua função seria a de agir como uma molécula estimulatória para a ativação das células T, sendo que a sua ligação por anticorpo monoclonal levaria a um aumento na produção de citocinas (particularmente IFN-γ) em um grupo de clones T CD4 estimulados pelos seus antígenos específicos [46].

Linfócitos em repouso, monócitos e leucócitos imaturos não apresentariam SLAM, ou a apresentariam em baixos níveis [44]. Monócitos ativados, por outro lado, apresentam SLAM, sendo que foi descrita a sua indução em monócitos incubados com o vírus do sarampo (o vírus induziria a expressão do seu receptor facilitando a infecção dessas células [47]). Da mesma forma, foi descrito aumento na expressão de SLAM em monócitos que foram induzidos a se diferenciar em células dendríticas mielóides imaturas, com concomitante aumento nos níveis de infecção nas células diferenciadas [48]. Essas células diferenciadas seriam mais facilmente infectadas pelas linhagens do vírus selvagem [21].

Por fim, o CD150 também regularia a produção de IL-4 e IL-13 pelas células T CD4+ [37,49] e a produção de IL-12, TNF-α e óxido nítrico pelos macrófagos [37], além de estar envolvido na geração de células B de memória, produção de anticorpos, ativação de células NK, inibição de células NK independente do MHC e no desenvolvimento de células NKT [49]. O CD150, da mesma forma que o CD46, sofre processo de supressão / internalização quando do seu contato com a hemaglutinina viral (tanto do vírus selvagem quanto do vacinal) [50, 51,52].

Se por um lado a descoberta de que a maior parte das linhagens do vírus selvagem usava o CD150 como receptor foi capaz de explicar a preferência do vírus do sarampo por células do tecido linfóide, por outro a sua capacidade de infectar células endoteliais [53] e outros tipos celulares, que não apresentam SLAM, levou à necessidade de se pesquisar a existência de outros receptores virais. Dentre os prováveis candidatos estão a moesina e o receptor da substância p [15], além do DC-SIGN – que aparentemente não favoreceria a entrada do vírus nas células que o possuem, mas atuaria como um facilitador da infecção de células vizinhas [54].

1.4 A patogênese do sarampo

O vírus do sarampo é transmitido pela via respiratória. Sua multiplicação, portanto, se iniciaria no epitélio respiratório e nas amígdalas [55] se espalhando, talvez com o auxílio de células dendríticas [56, 57,58], monócitos e macrófagos [57], para as células epiteliais do timo (causando lesão cortical significativa [59]), células do epitélio oral [60], da pele [60,61], dos pulmões [61, 62, 63], endoteliais, do sangue, linfonodos, baço, fígado, conjuntivas e superfícies mucosas dos tratos gastrointestinal, respiratório e urinário [1]. A célula primariamente infectada no sangue seria o monócito [64], mas experimentos *in vitro* mostraram que o vírus do sarampo pode também se multiplicar em linfócitos T e B de sangue periférico [65,66] e em células dendríticas [67], sendo capaz inclusive de induzir infecção persistente em linfoblastos [65,68]. Outro papel importante das células dendríticas na patogênese da doença seria o seu uso, pelo vírus do sarampo, como "cavalo de tróia", transportando-o do trato respiratório superior para os tecidos linfáticos onde, após um ciclo de replicação, o vírus se espalharia [19].

O linfotropismo do vírus do sarampo e sua natureza imunossupressora seriam em parte explicados pela expressão freqüente do receptor CD150 na superfície dessas células [37], sendo que as células ativadas apresentariam maior capacidade de suportarem a multiplicação viral que as em repouso [47, 66, 67, 68, 69]. O acometimento das células do estroma da medula óssea também foi descrito, levando ao comprometimento do desenvolvimento das células tronco hematopoiéticas [70]. Células polimorfonucleares, por outro lado, parecem não ser susceptíveis [61, 65]. Estudo em macacos mostrou que essa células expressariam baixos níveis de SLAM [71].

Apesar do antígeno viral não ter sido encontrado no cérebro de pacientes com sarampo agudo [61] e de ser discutível a sua presença em pacientes com encefalomielite pós-sarampo [61,72], sua reprodução estava aumentada em neurônios e em células da glia de pacientes com panencefalite esclerosante subaguda [61]. Acredita-se que uma mutação na cauda citoplasmática da proteína de fusão viral seja talvez a responsável pela propagação mais eficiente do vírus no cérebro desses pacientes, uma vez que a fusão entre as células seria a principal forma de disseminação viral no sistema nervoso central (SNC) [73]. Se por um lado a infecção viral do SNC parece ser de ocorrência pouco comum, foi notada pleocitose no líquor em cerca de 30% dos pacientes com sarampo agudo, sendo que 15% também apresentavam anticorpos no líquor contra o vírus do sarampo [74].

Contrário ao senso comum de que, com o surgimento da resposta imunológica e com a resolução dos sintomas haveria um clareamento total do vírus, estudo prévio mostra que talvez não seja este o caso [75].

1.5 O vírus do sarampo e o sistema imune

1.5.1 A imunossupressão induzida pelo vírus do sarampo selvagem

Há cem anos Von Pirquet descreveu que crianças portadoras de sarampo apresentavam perda temporária da capacidade de responder ao teste tuberculínico [76]. Tal relato é considerado um dos primeiros a demonstrar a capacidade imunossupressora do sarampo. Nesta mesma época já se havia notado que pacientes com sarampo agudo podiam apresentar remissão de sintomas diagnosticados como glomerulonefrite [76]. Hoje sabemos que a mortalidade associada ao sarampo estaria relacionada à capacidade do vírus em suprimir respostas imunes [19], favorecendo o surgimento e agravando o curso de infecções oportunistas, que são também complicadas por fatores sócio-econômicos como a desnutrição [2]. Essa imunossupressão poderia causar infecções recorrentes até dois anos após a infecção aguda pelo sarampo [77]. Dentre as infecções secundárias graves mais comuns estariam as pneumonias [78, 79, 80] (por *Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus*, *Haemophilus*, *Neisseria* etc.).

O vírus do sarampo, como dito anteriormente, pode infectar produtivamente linfócitos T e B [65, 68] (além de monócitos [65, 68, 81] – com diminuição da produção de

IL-12 [82] – e células dendríticas [19]), resultando em uma linfopenia que acometeria principalmente as células T [2, 19, 69], com diminuição [83] (ou manutenção [84]) da relação CD4/CD8. Outros autores, entretanto, acreditam que os linfócitos B seriam um alvo preferencial [77], com relato de diminuição persistente dessas células após a doença [29, 85, 86]. Permar et al, por outro lado, relataram diminuição no percentual de células T (CD4+ ou CD8+) virgens durante a doença, mas com aumento no percentual de células T de memória [87]. Foi também descrito que a multiplicação viral seria mais importante quanto maior o grau de ativação celular [88]. Desta forma, os leucócitos ativados por estímulos antigênicos seriam mais susceptíveis à infecção pelo vírus. Assim, em pacientes com tuberculose (TBC) quiescente, os linfócitos sensíveis à tuberculina seriam mais susceptíveis à infecção e conseqüente destruição, favorecendo a reativação da doença [68].

Apesar da viremia vir geralmente acompanhada de linfopenia significativa, esta parece ser decorrente da apoptose induzida pelo vírus e não de altos níveis de multiplicação viral [29].

A maior gravidade da linfopenia foi notada em pacientes portadores do HLA-AW32 [89]. Uma maior diminuição no número de células T CD4+, por sua vez, foi associada com desnutrição e com maior gravidade da doença [1].

Apesar do real motivo da linfopenia não ter sido ainda completamente esclarecido, sabe-se que quanto mais baixos os valores de linfócitos, pior o prognóstico da doença. Crianças com linfócitos totais menores que 2.000/mm^3 apresentavam não apenas maior morbidade, mas também maior mortalidade que aqueles com valores superiores [78]. Dentre as causas mais prováveis da linfopenia estariam não apenas a infecção e lesão celular direta pelo vírus, mas também a indução de apoptose e a migração celular aberrante das células T [15]. Desta forma, foi notado que linfócitos isolados de crianças na fase aguda do sarampo e colocados em cultura entravam mais freqüentemente em apoptose. Nesses pacientes, havia aumento na expressão de Fas (receptor associado à apoptose) tanto em linfócitos T CD4+ (auxiliares) quanto em linfócitos T CD8+ (citotóxicos), sendo mais significativo nos linfócitos T auxiliares [83]. Esse aumento no percentual de células apoptóticas persistia até seis meses após a infecção aguda [90]. Mesmo em modelos experimentais como culturas de células dendríticas humanas [58, 67], culturas de células epiteliais tímicas humanas [91], culturas de células VERO [92] ou na infecção de ratos

transgênicos cuja expressão do receptor CD46 mimetizava a localização e a quantidade encontradas em humanos [93], foi descrito aumento no percentual de células apoptóticas. Em culturas mistas de células dendríticas e células T, foi também observado aumento na apoptose das primeiras mediado pelo Fas [94]. O aumento nos níveis de Fas, entretanto, não foi observado em todos os estudos [90].

Além dos níveis de Fas, outros marcadores de apoptose foram descritos. Ohada e cols notaram não apenas aumento na expressão de Fas em linfócitos em repouso de pacientes no dia do *rash* cutâneo e nos dias subseqüentes, mas também aumento do TRAIL (ligante indutor da apoptose relacionado ao fator de necrose tumoral) e do seu receptor, principalmente antes do surgimento do *rash* [29]. Além desses marcadores, Okada e cols encontraram, em seus pacientes, aumento nos níveis de interferon gama (IFN-γ) plasmático e aumento nos níveis séricos de IL-18, responsável pela indução da apoptose em várias doenças infecciosas [85]. Vuorinen e cols, por sua vez, além de relatarem aumento da expressão de Fas em células mononucleares de sangue periférico, também discreveram ativação da granzima B e da cascata da caspase. O uso de inibidores de caspase foram, desta foram, capazes de levar a diminuição do percentual de células T apoptóticas [95].

Modelos experimentais também demonstraram o aumento de TRAIL induzido pelo vírus do sarampo. Vidalain e cols detectaram aumento de TRAIL na superfície de linfócitos T CD3+ e de monócitos, concomitante ao aumento de TRAIL intracelular em células dendríticas [96]. Desta forma, o aumento na expressão de TRAIL pelas células dendríticas infectadas [19, 57, 96,97] contribuiria para a morte celular dos linfócitos T.

A imunossupressão causada pelo vírus do sarampo, entretanto, não ocorreria somente devido ao aumento na morte celular. Apesar da ativação por mitógenos dos linfócitos T e B estar mantida, a sua proliferação estaria comprometida após a infecção pelo vírus do sarampo, uma vez que ocorreria um bloqueio na transição G0-G1 [91, 98, 99 ,100], ou na fase G1 [77, 88, 101,102], com conseqüente comprometimento da progressão do ciclo celular e da entrada na fase S [1,103]. A diferenciação dos linfócitos B em células produtoras de imunoglobulinas estaria também prejudicada [88].

Em indivíduos normais, a proteína p27 agiria como inibidor dos complexos ciclina – CDK (quinase dependente da ciclina), responsáveis pela progressão do ciclo celular. Após estímulos mitogênicos, a p27 sofreria um processo de degradação que resultaria na

progressão do ciclo. Após a infecção com o vírus do sarampo, o comprometimento da degradação desta proteína seria um dos principais fatores que comprometeriam a divisão celular [98, 102,104]. Mesmo vírus inativados com luz UV manteriam a capacidade de bloquear o ciclo celular [98, 102].

A expressão das ciclinas D3 e E, que controlam positivamente a entrada na fase S do ciclo celular, também estaria diminuída [98] e genes responsáveis pela síntese protéica e pela fosforilação oxidativa foram inibidos [97].

A importância da interleucina 2 (IL-2) e dos sinais transmitidos pelo seu receptor na sobrevivência e na progressão para a fase S das células T está bem estabelecida. Avota e cols estudaram a transmissão desses sinais estimulatórios em células infectadas pelo vírus do sarampo. Foi evidenciado bloqueio na transmissão dos sinais da IL-2 com conseqüente bloqueio no ciclo celular em G1, associado ao comprometimento da ativação da quinase AKT (proteína relacionada a sinais anti-apoptóticos) [105].

A infecção pelo vírus do sarampo de células dendríticas (DCs) também parece contribuir para o processo de imunossupressão. Já foi relatado que a infecção das DCs leva à perda da sua capacidade de estimular a proliferação de células T CD4+ virgens em culturas mistas de células. A presença de 10 células dendríticas infectadas é capaz de inibir o efeito estimulatório de 10^4 células dendríticas não infectadas. Mesmo as células infectadas submetidas à radiação UV mantêm a sua capacidade inibitória, levando-se à conclusão que proteínas virais expressas na superfície dessas células poderiam ser as responsáveis por esse efeito [106].

As DCs infectadas também apresentam diminuição da sua viabilidade [106]. Schnorr e cols, por sua vez, notaram que a infecção dessas células foi capaz de interferir fortemente com a proliferação dependente de mitógenos de linfócitos de sangue periférico *in vitro* [107]. A infecção pelo vírus selvagem levou a uma maior capacidade de inibição que a infecção pelo vírus vacinal [2, 107], provavelmente devido ao maior tropismo da linhagem selvagem pelas células dendríticas [2, 21, 22].

A interação de células dendríticas infectadas com linfócitos T CD4+ leva também à formação de sincício, onde o vírus se multiplica massivamente. Foi observada concomitante diminuição na capacidade de produzir IL -12 pelas DCs infectadas [108].

Essa diminuição na produção de IL-12 poderia ser um fator chave contribuindo para a polarização do tipo 2 observada nos pacientes infectados [19].

Bieback e cols, por sua vez, mostraram que o uso do vírus selvagem em experimentos *in vitro* levou, na realidade, ao aumento na produção de IL-12. Esse aumento seria secundário à estimulação do receptor *toll-like* 2 (TLR-2) pela hemaglutinina do vírus selvagem (que não ocorria com a linhagem vacinal). Na realidade, havia aumento na expressão de vários genes responsivos ao estímulo via TLR-2 como o da IL-1α, IL-1β, e IL-6 (citocina pro-inflamatória). A propriedade única da hemaglutinina do vírus selvagem em ativar sinais dependentes do TLR-2 poderia contribuir, conforme descrito anteriormente, não somente para a ativação imune, mas também para a disseminação viral e patogenicidade, pela indução do aumento da expressão do seu receptor em monócitos [22]. A infectibilidade de monócitos e células dendríticas imaturas, que geralmente não expressam SLAM, seria, portanto, explicada pelo aumento na sua expressão após a ativação do TLR-2 [19].

Em pacientes infectados pelo sarampo, por outro lado, não foram notadas alterações nos níveis de IL-12 sérica [85]. Os níveis de IL-8 e de IL-1β, por sua vez, encontraram-se aumentados na fase aguda do sarampo [109], sendo que os de IL-1β mantiveram-se elevados na fase de convalescença [109,110].

A infecção pelo vírus do sarampo foi também capaz de alterar a expressão de vários genes em células mononucleares de sangue periférico de crianças infectadas. Entre os genes suprimidos encontram-se alguns envolvidos na transcrição, transdução de sinal e em respostas imunes como o responsável pela produção de IL-6, ou aqueles associados à expressão de receptores celulares como o IL-4R, IL-6R, IL-7R, IL-27RA CCR2 e CCR7. Entre os genes cuja expressão encontrava-se aumentada estão os das moléculas imunes IL-1β, fator da necrose tumoral α, a molécula de adesão intercelular 1 (ICAM-1), e as quimiocinas MIP-1β, GRO-β e IL-8 [109]. Experimentos em cultura de células de tecido linfóide infectadas pelo vírus do sarampo também mostraram aumento na expressão das quimiocinas MIP-1α, MIP-1β e RANTES [111].

As células NK também apresentaram diminuição na sua atividade durante o sarampo agudo. Esse comprometimento na função das NK estava presente mesmo três semanas após o surgimento do *rash* cutâneo, podendo ser um dos fatores contribuintes à

maior susceptibilidade a infecções secundárias observadas durante o sarampo [112]. Os baixos níveis de TNF-α nos pacientes com sarampo poderiam estar associados à diminuição da atividade das NK [110].

Muitas são as citocinas que apresentam alterações significativas na sua expressão após a infecção pelo sarampo. Além das já anteriormente descritas, foi também notada diminuição de 10 a 80 vezes na síntese de interferon α e β (IFN-α e β) por células mononucleares de sangue periférico de pacientes infectados; citocinas essas cruciais na resposta antiviral precoce. A supressão dos IFN-α e β poderia levar à lentificação na resposta imune inata, permitindo a disseminação viral precoce [113]. A inibição da síntese dos interferons α e β parece ser secundária ao bloqueio da sinalização dos receptores *toll-like* 7 e 9, levando também à maior permissibilidade desses indivíduos a infecções por outros patógenos [114].

As respostas transcricionais induzidas pelos IFN-α e β também estariam bloqueadas pela ação da proteína viral V, que interferiria com a translocação nuclear da STAT [115].

Os níveis de IL-10 também estariam aumentados [85]. Sua elevação pôde ser detectada por várias semanas no plasma de crianças infectadas [116]. Acredita-se que esses níveis elevados poderiam potencialmente contribuir para uma imunidade celular defeituosa e respostas de hipersensibilidade diminuídas após o sarampo [116].

A IL-2, citocina do tipo 1, encontrava-se aumentada no fase do *rash* viral e por pouco após o seu desaparecimento. Já citocinas do tipo 2, como IL-4 e IL-13, também se encontravam elevadas em crianças infectadas, sendo que os valores de IL-4 persistiram elevados por mais de um mês após o surgimento do *rash* [116,117]. Desta forma, ocorreria mudança na resposta imune durante a infecção, com uma resposta predominantemente do tipo 1 na fase inicial da doença, que mudaria para uma resposta predominantemente do tipo 2 na fase de convalescença [117].

Experimentos *in vitro* mostraram também aumento na expressão de IL-4, além de diminuição na expressão de IFN-γ. Os efeitos supressores da IL-4 nos linfócitos T CD4+ e nos macrófagos poderiam contribuir para a supressão da hipersensibilidade tardia e diminuição na ativação dos macrófagos, observadas no sarampo. A neutralização *in vitro* da IL-4 foi capaz de corrigir parcialmente o defeito na linfoproliferação notado nos pacientes [117].

Sabe-se que o CD150 é uma molécula co-estimulatória importante na ativação das células T e B e que a sua ligação induz tanto a proliferação das células como a produção do IFN-γ [39]. A internalização do CD150, secundária à sua ligação com a hemaglutinina viral [52] e a destruição das células CD150+, observada nos pacientes infectados, poderiam favorecer resposta imune do tipo 2 prolongada observada nos pacientes [44, 51], que se exacerbada pode contribuir para o estado de imunossupressão causada pelo vírus [39]. O processo de apresentação antigênica poderia também estar comprometido, uma vez que células dendríticas ativadas geralmente apresentam SLAM em sua superfície [44], levando a um maior efeito citopático pelo vírus [48]. Desta forma, a infecção das células apresentadoras de antígeno levaria à interferência na maturação e na função das mesmas, afetando conseqüentemente a ativação das células T [57].

A infecção pelo sarampo parece também contribuir para a disfunção de órgãos responsáveis pela produção e maturação de células imunes. A combinação de um meio de citocinas supressor na medula óssea, associada à depleção tanto de populações do estroma quanto de células CD34+, poderia prejudicar significativamente a repopulação dos precursores linfóides após a linfopenia induzida pelo vírus [70].

As células epiteliais tímicas, por sua vez, são não somente infectadas [61], mas também sofrem com a apoptose induzida pelo sarampo [91], podendo então contribuir para distúrbios na maturação normal das células T [61]. A apoptose dessas células levaria à liberação da proteína N viral, que se ligaria a outras células epiteliais tímicas, levando à inibição da proliferação celular espontânea, devido ao bloqueio do ciclo celular em G0 / G1. A proteína N se ligaria também às células T ativadas levando à supressão da sua proliferação [91].

O comprometimento de células tímicas não foi observado somente em experimentos *in vitro*. Crianças que evoluíram para o óbito devido ao sarampo apresentavam precocemente na doença grandes sincícios de timócitos que progrediram para a destruição citoplasmática e nuclear. Em estágios mais avançados, foi notada perda total do córtex tímico, que poderia estar ausente mesmo dois meses após a infecção aguda. A lesão, entretanto, não se restringiu ao córtex, sendo observada pequena diminuição tanto na medula quanto nos corpúsculos de Hassal durante a fase aguda da doença. A destruição do

timo seria, portanto, um dos fatores que contribuiria para a depressão da imunidade celular durante o sarampo [59].

Apesar da destruição tímica, foi recentemente descrito aumento nos níveis de TRECs (*T cell receptor excision circles*) em linfócitos T CD8+ nas fases iniciais da doença e aumento mais tardio nos níveis de TRECs em linfócitos T CD4+, que ocorriam de forma sustentada por um mês após a fase aguda. As células TREC+ seriam aquelas que teriam migrado recentemente do timo para a circulação periférica [87].

As proteínas virais também seriam capazes de contribuir para a inibição da proliferação celular, imunodeficiência celular e humoral detectadas durante a doença.

A proteína N, por exemplo, foi capaz de inibir a produção de IL-12 por monócitos e macrófagos [25]. Nas células apresentadoras de antígeno, a proteína N afetaria não somente o "*priming*" dos linfócitos T CD8+ virgens durante sua sensibilização, mas também o desenvolvimento da fase efetora após o contato secundário com o antígeno [108]. A proteína N também inibiria a proliferação de vários tipos celulares, induzindo a interrupção do ciclo celular em G1[103]. Da mesma forma, ela foi capaz, ao se ligar ao seu receptor FCγRII, de inibir a produção de imunoglobulinas pelos linfócitos B [118], além de inibir a proliferação das células T antígeno específicas [108]. Na realidade, a proteína N teria dois domínios, chamados de N_{CORE} (aa 1-400) e N_{TAIL} (aa 401-525). O N_{CORE} se ligaria ao receptor FCγRIIB1 levando à apoptose mediada pela caspase 3 e o N_{TAIL} se ligaria ao receptor da proteína N (NR), induzindo o bloqueio do ciclo celular nas fases G0/G1 [100].

Conforme dito anteriormente, a proteína C estaria envolvida na prevenção da morte celular, se relacionando à infecção persistente pelo vírus do sarampo [26].

A proteína P, por outro lado, inibiria a fosforilação de STAT1 (transdutor de sinal e ativador de fatores de transcrição) bloqueando a sua translocação nuclear e contribuindo com a evasão imune [119].

O complexo glicoproteico do envelope viral também parece estar implicado no processo de imunossupressão induzido pelo sarampo [2, 19, 103, 106, 108, 120 ,121], inibindo a proliferação celular após o seu contato com a superfície das mesmas. Schlender e cols mostraram que o complexo das duas glicoproteínas do envelope viral (proteínas H e F) contribui para a supressão da proliferação observada durante o sarampo [120]. Weidmann e cols, por sua vez, relataram que mais importante que a fusão do complexo glicoproteico

com a célula alvo, o fator primordial envolvido no processo de imunossupressão seria a clivagem proteolítica da proteína F [122].

Com relação à reação cutânea de hipersensibilidade tardia, foi encontrada redução da resposta ao dinitroclorobenzeno, mesmo após seis semanas do início da doença [86]. Essa perda poderia estar relacionada tanto à diminuição do número de linfócitos T, que pôde ser notada mesmo após quatro semanas do início da doença [69], quanto aos efeitos supressores da IL-4 sobre os linfócitos T CD4+ [1]. A proteína N viral poderia também estar associada à perda da hipersensibilidade tardia [108].

Além da supressão das reações de hipersensibilidade tardia, o vírus do sarampo seria também capaz de comprometer as reações proliferativas a estímulos antigênicos e as respostas imunológicas tanto humorais quanto celulares.

Uma das razões para a inibição da proliferação das células T a mitógenos *in vitro* (que atingiria 50-90% [98, 101]) seria a produção inadequada de IL-2, pois, quando a mesma era adicionada às culturas de linfócitos infectados, notava-se alguma melhora do quadro [1, 110]. A melhora na inibição da proliferação após a neutralização da IL-4 também foi descrita [117].

A proteína N viral também estaria envolvida na inibição da proliferação das células T antígeno-específicas [108]. Além disso, as células dendríticas infectadas também interferiram fortemente na proliferação dependente de mitógenos dos linfócitos de sangue periférico, transmitindo um sinal inibitório que era maior quando infectadas com a linhagem selvagem [2, 107]. Por outro lado, o vírus inativado pela luz UV também foi capaz de inibir a proliferação das células T [67].

A proliferação das células B também se encontraria suprimida (em até 90%), ocorrendo prejuízo na imunidade humoral, uma vez que haveria um comprometimento na sua diferenciação em células secretoras de imunoglobulinas [1, 77, 88]. Desta forma, pacientes que produziam baixos níveis de anticorpos contra o vírus do sarampo apresentaram maior mortalidade e também maior morbidade [78]. Foi também constatado que em pacientes desnutridos a imunidade humoral encontrava-se ainda mais deprimida, uma vez que houve uma menor resposta à vacinação contra o meningococo naqueles com comprometimento nutricional [9].

O sarampo é uma doença altamente catabólica, associada com diminuição da aceitação das dietas, perdas gastrointestinais aumentadas e rápida perda de peso [123]. A perda proteica fecal e a má absorção que surgem com a doença podem também contribuir significativamente para o desenvolvimento da desnutrição, sendo que a diarréia crônica pode persistir por várias semanas após a fase aguda. As infecções secundárias podem também prolongar o período de debilidade, notando-se muitas vezes o aparecimento de Kwashiorkor nesses pacientes [124]. A desnutrição, desta forma, poderia estar associada à imunossupressão notada durante e após a infecção aguda pelo sarampo, uma vez que crianças desnutridas tem uma diminuição no número de linfócitos T funcionantes, diminuição da resposta imune a lipopolissacárides, depressão significativa na capacidade de opsonização, deficiência na produção de IgG2 e IgG4 contra microorganismos encapsulados e inclusive involução do timo [125].

A supressão da imunidade celular ocorreria durante semanas a meses após o clareamento viral, sendo a principal causa das infecções secundárias e da mortalidade pelo sarampo [70]. Joseph e cols, por sua vez, propuseram que o prejuízo na imunidade celular observada no sarampo poderia representar mais um comprometimento específico do sistema imune que uma ampla imunossupressão induzida pela doença, uma vez que o vírus se multiplicaria (e depletaria) mais eficientemente as células ativadas (como os leucócitos ativados por estímulos antigênicos) [68].

Apesar da imunossupressão causada pelo sarampo, acredita-se que a resposta imune desenvolvida pelo hospedeiro seria capaz de levar ao clareamento viral; por outro lado, estudos têm demonstrado que a infecção persistente não só é possível [65, 66], como pode ser muito mais comum do que se esperava, uma vez que necrópsias de pacientes que evoluíram ao óbito por causas não relacionadas ao sarampo mostraram presença do RNA viral em mais de 45% dos casos [75]. Foi também detectado RNA viral em 20% de espécimes cerebrais em necrópsias de adultos sem relato de doença neuronal pelo sarampo [16].

1.5.2 A imunossupressão induzida pelo vírus do sarampo vacinal

A linhagem vacinal poderia também levar a um estado de imunodepressão, mas em grau menor que a causada pela linhagem selvagem [1, 85, 126]. Foram identificadas, desta

forma, supressão da resposta cutânea a testes de hipersensibilidade tardia [1, 126], linfopenia transitória, diminuição da proliferação de linfócitos estimulados por antígenos e por mitógenos e alterações na produção de citocinas [1]. Nas células dendríticas infectadas pelo vírus vacinal foi observada inibição da produção de IL-12 [67], que poderia contribuir para uma polarização da resposta imune do tipo 2 [67] após a vacinação. A infecção de monócitos com uma linhagem vacinal também resultou em diminuição na produção de IL-12. Uma redução semelhante foi obtida com o uso de anticorpos monoclonais contra o CD46 (um dos receptores do vírus do sarampo) [82].

1.5.3 Resposta imune induzida pelo vírus do sarampo selvagem

O vírus do sarampo estimula uma resposta imune duradoura, apesar da imunossupressão observada durante a doença. Acredita-se que a infecção sistêmica causada pelo vírus, com conseqüente multiplicação viral disseminada nos tecidos linfóides, resultaria em uma estimulação antigênica eficaz, favorecendo essa resposta permanente [127]. Assim, apesar da leucopenia e da linfopenia observadas na fase aguda da doença [84], que levariam a uma maior morbimortalidade [78], seria induzida, concomitantemente ao *rash*, uma resposta celular potente, que promoveria o clareamento viral [70]. É sabido que, uma semana após o *rash* cutâneo, não se consegue mais isolar o vírus em linfócitos de pacientes infectados [69].

Na resposta imunológica contra o sarampo, a imunidade celular parece ter papel preponderante, sendo essencial para a superação da doença [103]. Conseqüentemente, pacientes com imunodeficiências humorais geralmente se recuperam bem do sarampo, enquanto aqueles com imunodeficiências celulares apresentam grandes riscos de complicações fatais [77].

O vírus seria eliminado das células mononucleares de sangue periférico através de uma resposta imune celular específica e tanto os pacientes quanto os vacinados apresentam respostas protetoras duradouras [2]. Clones de linfócitos T CD4+ e T CD8+ direcionados às proteínas hemaglutinina, fusão, matriz e nucleoproteína são detectados, ocorrendo predominância das células T CD8+ [77], indicando um papel primordial das mesmas na recuperação da doença [77, 128]. Em humanos após a doença, 77% apresentavam respostas T citotóxicas à proteína de fusão (F), 69% à hemaglutinina (H) e 50% à nucleoproteína (N).

Essa resposta se relacionou à gravidade da doença, sendo que os sintomas mais graves ocorreram naqueles com respostas mais fracas [129].

A resposta imune gerada por linfócitos T citotóxicos foi também evidenciada em macacos expostos ao vírus selvagem [130].

Nos homens, os linfócitos citotóxicos aumentaram mais rapidamente na circulação após a doença e os níveis de CD8 solúvel eram maiores que os encontrados em mulheres, sendo talvez uma das razões para a menor mortalidade em indivíduos do sexo masculino [83].

Tanto os linfócitos T CD4+ quanto os T CD8+ encontram-se ativados durante o *rash* [1, 117]. O perfil fenotípico de ambas as células mostra diminuição no percentual de células virgens (RA+ 62L+) e aumento no percentual de células ativadas (HLA-DR+, CD25+ ou CD69+) [87].

Estudo realizado em crianças infectadas evidenciou aumento inicial na produção de citocinas do tipo 1 (IL-2 pelas células CD4+ e de IFN-γ pelas células CD8+) que foram substituídas subseqüentemente por citocinas do tipo do tipo 2 (IL-4 e IL-13). A resposta inicial do tipo 1 levaria ao clareamento viral, enquanto a resposta do tipo 2 suportaria o desenvolvimento de anticorpos específicos contra o sarampo131]. Essa mudança no padrão do tipo 1 para 2 poderia, entretanto, ser secundária à destruição das células CD150+ pelo vírus do sarampo, uma vez que as células do tipo 1 tendem a expressar mais CD150 que as células do tipo 2 [44].

Houve também aumento de outras citocinas no decorrer da infecção pelo sarampo. O receptor solúvel da IL-2 encontra-se aumentado na fase aguda da doença (denotando ativação dos linfócitos T) [110,132], ocorrendo correlação positiva dos seus níveis com os níveis de neopterina [132]. Também durante a fase aguda foi notada diminuição nos níveis de TNF-α [110]. Na fase de convalescença haveria ainda níveis baixos de TNF-α, mas ocorreria normalização dos níveis do receptor solúvel de IL-2 e aumento na produção de IL-1β, denotando uma ativação *in vivo* dos monócitos [110].

A ativação dos linfócitos T CD4+ persistiu por várias semanas após o desaparecimento do *rash* [117], promovendo ajuda aos linfócitos B para a geração de anticorpos específicos contra o sarampo [77]. De fato, os anticorpos surgiram

concomitantemente ao *rash*, sendo que a viremia aparentemente se encerra com o seu aparecimento [133].

O surgimento da resposta imune contra o sarampo seria então marcado pelo aparecimento de linfócitos T específicos, pela infiltração de células mononucleares em áreas de multiplicação viral e pelo aparecimento de anticorpos contra o vírus [19]. O primeiro anticorpo detectado após a infecção é do tipo IgM, ocorrendo depois uma mudança no isotipo para IgG (principalmente IgG1 e IgG4) [1, 7]. Apesar do anticorpo mais abundante ser aquele contra a proteína N e apesar de serem produzidos anticorpos contra a maioria das proteínas virais [1], os únicos anticorpos neutralizantes e protetores seriam aqueles direcionados contra as glicoproteínas de superfície H e F [77].

A IgA sérica aumentaria nos dois primeiros dias depois do *rash*, com pico após uma semana, permanecendo detectável por meses após a recuperação da doença. Quanto à IgE, não seriam detectáveis anticorpos específicos contra o sarampo, apesar de haver aumento policlonal que parece preceder a resposta imune específica e que teria seu pico uma semana após o início do *rash* [77]. Em pacientes com encefalomielite pós-sarampo os níveis de IgE permaneceram persistentemente elevados, comparados com os pacientes com complicações pulmonares ou com aqueles sem complicações [134].

Experimentos com macacos também evidenciaram a presença de anticorpos neutralizantes, que surgiram entre os dias 7 e 14 após a infecção [135]. Esses anticorpos neutralizantes seriam importantes na prevenção da infecção sistêmica, mas seria a imunidade celular que estaria relacionada com a recuperação da infecção e que também preveniria o surgimento de doença mais grave [130].

Durante a infecção pelo sarampo foi notado aumento no número e na ativação das células NK [85]. As células NK poderiam chegar a atingir 80% do total de linfócitos, sendo descrito aumento na expressão de IFN-γ pelas mesmas [29].

1.5.4 Resposta imune induzida pelo vírus do sarampo vacinal

Após a vacinação, os principais anticorpos encontrados foram os do tipo IgG1 e IgG4, enquanto IgG2 e IgG3 parecem não ser detectados quando da reexposição ao vírus [77]. Os níveis de IgG e IgM nos vacinados, entretanto, foram 100 vezes menores que os encontrados nos pacientes infectados pelo vírus selvagem [85]. Pode-se também detectar

uma resposta celular, com aumento de linfócitos citotóxicos, menor em pacientes vacinados do que naqueles que sofreram infecção pelo vírus selvagem [136]. Foram notadas respostas citotóxicas contra a proteína de fusão e contra a hemaglutinina em 70% dos casos e contra a proteína N em 50% dos casos [129].

A resposta imune gerada por linfócitos T citotóxicos foi também evidenciada em macacos vacinados, estando presente mesmo nove meses após a vacinação inicial [130]. Desta forma, pacientes com defeitos na geração de respostas citotóxicas ao sarampo após a vacinação poderiam apresentar maior susceptibilidade à infecção pelo vírus selvagem [136].

Com relação às células NK, não foi notado aumento no seu número nem na sua ativação nos pacientes vacinados [85].

1.6 Manifestações clínicas

A transmissão do sarampo é bastante elevada, ocorrendo pela via respiratória através do contato com perdigotos [16]. Seu vírus pode permanecer ativo por até duas horas no ar ou nas superfícies infectadas [4]. Clinicamente é descrito inicialmente um período de latência, que varia de 10 a 14 dias, que é seguido por um período prodrômico, que dura cerca de dois a três dias, durante o qual ocorrem febre, coriza, tosse e conjuntivite [1]. A febre, na verdade, duraria de um a sete dias [4]. Durante o período prodrômico o paciente pode também apresentar o sinal de Koplik, que são pontos vermelho-brilhantes com uma pequena mancha branco-azulada no centro, que se tornam visíveis principalmente na mucosa oral [1]. Os pontos de Koplik estão mais evidentes nos dois dias que antecedem e nos dois dias que sucedem o surgimento do *rash* [60]. O *rash* maculopapular típico ocorre apenas no fim da doença. O período de maior infectividade ocorreria de quatro a cinco dias antes do surgimento do *rash*, até 4 dias após seu aparecimento [1, 4]. O vírus seria encontrado em secreções por um período não superior a 10 dias em pacientes imunocompetentes [137]. Em países desenvolvidos a criança geralmente se recupera uma semana após o surgimento do *rash* devido a uma resposta imune vigorosa [77].

O vírus pôde ser identificado através de RT-PCR de amostras de fluido oral e sangue seco. No entanto, as amostras de *swabs* nasofaringeanos, urina e células

mononucleares de sangue periférico apresentaram a maior sensibilidade para a detecção viral [28].

A linfopenia foi um dos achados laboratoriais mais comuns durante o sarampo, provavelmente associada à indução de apoptose pelo vírus. A gravidade e a duração da linfopenia foram dependentes da idade, sendo mais importantes em lactentes menores de um ano, em adolescentes e em adultos [29].

Em pacientes portadores de imunodeficiência humoral não foram notados maiores danos secundários ao sarampo [1, 137]. Naqueles com imunodeficiência celular (leucêmicos, HIV+, portadores de algumas imunodeficiências primarias), por outro lado, a doença pode ser progressiva, levando à pneumonia de células gigantes, à encefalite por corpos de inclusão e à morte [137]. Essas crianças podem não desenvolver o *rash*, que é uma manifestação da resposta imune do hospedeiro [137].

Nas crianças portadoras do HIV, apesar do curso da infecção pelo sarampo poder ser protraído, com tempo maior de eliminação viral na urina e em secreções [137], nota-se uma supressão temporária na multiplicação do HIV [16]. Estudo realizado por Moss e cols mostrou que a carga viral mediana do HIV durante a infecção aguda pelo sarampo era de 8.216 cópias/μl, enquanto um mês após a alta hospitalar alcançava 373.748 cópias/μl. Uma das causas aventadas para a queda na carga viral do HIV seria a destruição das suas células alvo (linfócitos, especialmente CD4+) observada durante a infecção pelo sarampo [138]. Em estudo realizado em culturas de tecidos, Grivel e cols notaram, além da destruição dos linfócitos T CD4+, um aumento nos níveis de citocinas inibidoras do HIV, principalmente RANTES, ligante natural do receptor CCR5 (que funciona como co-receptor durante a infecção pelo HIV) [111].

As complicações pelo sarampo geralmente surgem nas primeiras quatro a seis semanas após a infecção [7]. Entre as mais comuns se incluem diarréia [4, 16, 110,139], pneumonia [4, 16, 62, 63, 79, 110, 139] e encefalite [4, 16, 139], podendo ocorrer em cerca de 30% dos casos [16]. As otites também seriam freqüentes [4, 19]. Outras complicações descritas foram cegueira secundária a ulcerações na córnea [4, 140], sinusites [79], amigdalites [139], laringites [63], miocardites [139], pericardites [63], envolvimento pleural como empiemas e pleurites [63], anormalidades da função hepática [79, 139], apendicites [63], peritonites [63], alterações transitórias no eletroencefalograma [74], etc.

No sistema nervoso central, as complicações mais comuns são encefalomielite disseminada aguda, panencefalite esclerosante subaguda e encefalite por corpos de inclusão.

A encefalomielite disseminada aguda (EDA) é a complicação neurológica mais comum após o sarampo, ocorrendo em uma em cada 1.000 crianças maiores de dois anos [141]. A taxa de mortalidade é de 10 a 20% e a maioria dos sobreviventes desenvolve seqüelas neurológicas [141]. É uma doença desmielinizante inflamatória aguda perivenular [141] auto-imune, que está associada a uma resposta imune à proteína básica da mielina [1,141], geralmente não se encontrando o vírus no cérebro desses pacientes [1, 133, 141] (a ausência de vírus detectável nesses pacientes é na realidade controversa [72]). É a doença desmielinizante mais comum em humanos [141]. A tomografia computadorizada pode evidenciar áreas de baixa densidade no cérebro dos pacientes [142]. A EDA ocorre concomitantemente ou logo após a infecção aguda, seus sintomas tendem a se desenvolver de forma súbita, sendo freqüentes a confusão mental e as convulsões [141], podendo se associar à surdez [16]. Os achados liquóricos mais comuns são proteinorraquia e pleocitose linfocítica leves, além da presença da proteína básica da mielina [141].

A panencefalite esclerosante subaguda (PES), por sua vez, é de ocorrência mais rara, acometendo 1/100.000 pacientes [1]. Acontece mais freqüentemente em pacientes que tiveram a infecção pelo sarampo quando muito jovens [1] e estaria associada à infecção de células neuronais e da macróglia [2, 7]. Foi detectada também infecção de macrófagos, tendo sido aventada a possibilidade que os mesmos poderiam disseminar o vírus nos cérebros dos pacientes, principalmente para os astrócitos perivasculares [143].

A PES é uma doença de evolução lenta e progressiva que pode ocorrer meses a anos após a infecção aguda. É caracterizada por mudanças de comportamento, ataxia, crises convulsivas, deterioração mental e motora [144] levando geralmente à morte do paciente [16]. Em raros casos pode haver melhora significativa com sobrevida quase normal por vários anos. A doença tem quatro estágios que variam em sua duração. I- Sinais motores (duração de um a dois meses). II- Coma e opistótono (duração de dois a três meses). III- Mutismo e perda da função cérebro-cortical (duração de um a quatro meses). IV- Mioclônus (duração de meses a anos) [144].

Patologicamente, o cérebro desses pacientes é descrito como "decorticado", havendo inflamação maciça, ativação glial, perda neuronal e lesão da barreira hematoencefálica [16]. Os genótipos mais freqüentemente associados à PES são C1, D1, E e F [28], sendo descrita alteração freqüente na cauda citoplasmática da proteína F dos vírus que se espalham no cérebro desses pacientes [73].

Os achados laboratoriais mais comuns são: 1) Mudanças no eletroencefalograma como períodos bem definidos de surtos periódicos de atividade de alta voltagem com intervalos entre os surtos de 3 a 5 segundos. 2) Níveis elevados de gama globulina e anticorpos contra o sarampo no líquor (úteis no estabelecimento do diagnóstico). 3) Evidências histológicas e de microscopia eletrônica com inclusões contendo nucleocapsídeos semelhantes aos dos da família *Paramyxoviridae* [144].

A encefalite por corpos de inclusão seria uma doença semelhante à panencefalite subaguda esclerosante, mas com sintomatologia precoce e progressão mais rápida [1]. É rara e afeta pacientes imunocomprometidos (particularmente os com imunodeficiências celulares) [137] semanas a meses após a infecção aguda [16].

A reativação [1] e a disseminação [68] da tuberculose e a remissão de doenças mediadas imunologicamente como a síndrome nefrótica, artrite reumatóide juvenil e púrpura trombocitopênica idiopática também foram descritas em associação ao sarampo [1]. Há relatos inclusive de regressão de linfomas de Hodgkin [145] e de Burkitt [146] associados à doença. A imunossupressão causada pelo sarampo favoreceria o estabelecimento e agravaria o curso de infecções oportunistas [2, 15], contribuindo também para a reativação de infecções persistentes, que seriam complicadas por fatores sócio-econômicos como a desnutrição. Assim, em países em desenvolvimento, as infecções pelo sarampo estariam associadas com alta morbimortalidade [2].

Além das complicações normalmente vinculadas à imunodeficiência desencadeada pelo sarampo, outras doenças vêm sendo associadas, apesar de muita controvérsia, com a persistência do vírus no hospedeiro – dentre elas a esclerose múltipla [16], doença de Paget [16], doença de Crohn [75], osteoesclerose [75], epilepsia [75], meningite crônica asséptica [75] e a hepatite auto-imune [75].

A morbimortalidade está associada à capacidade do sarampo em suprimir respostas imunes [19], é mais freqüente nas crianças desnutridas [4, 9], nos HIV+ [4], nos deficientes

em vitamina A [4], nos extremos de idade, naqueles com baixo nível sócio-econômico, onde não há acesso a cuidados médicos e quando a doença é introduzida em populações virgens [1]. A persistência viral também foi maior no grupo de crianças desnutridas [9]. A mortalidade média é de 1 a 5%, chegando a 25% nas populações com maiores níveis de desnutrição e acesso precário aos meios de saúde [4].

Em estudo realizado com crianças africanas foi evidenciado também que, além da maior mortalidade, havia maior incidência de sarampo no grupo de crianças cujos pais tinham baixa escolaridade; os casos eram também mais freqüentes durante a estação seca. Foram também descritas maior mortalidade em meninas [6, 147] e em crianças nos primeiros anos de vida [6, 63]. A morbidade [4, 140] e consequentemente a necessidade de terapia intensiva também foi maior nas crianças menores [147]. A patogenicidade estava também mais associada a fatores do hospedeiro que a determinantes virais [121], sendo que Coovadia e cols notaram que a linfopenia persistente por pelo menos 15 dias após o *rash* permaneceu como um bom índice preditivo de morbimortalidade e que todos os pacientes que morreram falharam em produzir anticorpos adequados contra o vírus [78]. Infecções secundárias, como as pneumonias, seriam uma das principais causas de mortalidade [62], sendo que o óbito tendia a ocorrer mais tardiamente (após a primeira semana, na maior parte dos casos) [63].

1.7 Tratamento

Levando-se em conta que a maior morbimortalidade do sarampo encontra-se associada à desnutrição e às infecções secundárias, torna-se evidente que os pilares do tratamento se baseiam no suporte nutricional, no tratamento da desidratação com soluções de reidratação oral e no tratamento das infecções secundárias com antibioticoterapia adequada [4].

O suporte nutricional adequado é essencial aos pacientes que sofrem de infecções agudas e crônicas [125] e deve ser promovido a todos os pacientes infectados com o sarampo que necessitem de internação hospitalar [123]. O aleitamento materno deve ser altamente encorajado aos lactentes e, nas crianças que recusarem a alimentação, deve-se considerar o uso de sonda nasogástrica [123]. No caso de pacientes com desnutrição

proteico-calórica a OMS recomenda dieta inicialmente freqüente e com baixo volume, com aporte calórico de 100 kcal/kg/dia. O aporte proteico inicial seria de 1 a 1,5g/kg/dia [148].

Pessoas expostas ao sarampo também poderiam ser protegidas com o uso de imunoglobulinas, que teria eficácia nos primeiros seis dias após a exposição inicial [77].

O uso de antiretrovirais não é feito rotineiramente, mas a ribavirina, que inibe o vírus do sarampo *in vitro*, poderia auxiliar na redução da gravidade dos sintomas [1]. Acredita-se que seu uso na forma de aerosol deveria ser considerado no tratamento das pneumonias primárias pelo vírus. Seu uso venoso foi bem tolerado em um estudo não controlado em pacientes adultos, sendo associado à melhora clínica da pneumonia viral [80].

O uso da vitamina A também tem sido preconizado pela Organização Mundial de Saúde (OMS) para crianças de países em desenvolvimento – duas doses com intervalo de 24 horas – uma vez que poderia prevenir o dano ocular e a cegueira, além de reduzir o número de mortes em 50% [4]. A associação da vitamina A com a imunidade provém de estudos anteriores à descoberta da sua estrutura. Estudos *in vitro* sugerem que os retinóides são reguladores importantes da diferenciação dos monócitos, além de estarem envolvidos na síntese de anticorpos contra antígenos dependentes das células T. A vitamina A também seria fundamental na manutenção da integridade do epitélio. Por outro lado, a suplementação da vitamina A em crianças levou a um aumento significativo na contagem total dos linfócitos e um aumento na proporção de células T CD4 virgens, além de aumento nos títulos de IgG de crianças com sarampo [149]. Sua deficiência está associada à maior gravidade do sarampo [92, 149], sendo seu uso preconizado na fase aguda da doença, pois seria capaz de reduzir a morbimortalidade [1, 92, 131] mesmo na ausência de evidência clínica da sua deficiência, levando a uma melhora no prognóstico [140, 147]. A suplementação crônica de vitamina A, por outro lado, parece não surtir muito efeito [6, 150].

1.8 Vacinação

A vacinação contra o sarampo é, seguramente, a forma mais eficaz de se controlar a doença. A falência em se manter altos níveis de imunidade na população, por outro lado, resulta na acumulação de indivíduos susceptíveis, criando condições que favorecem a

transmissão rápida de genótipos recentemente introduzidos[28]. Assim, o insucesso em se controlar o sarampo decorre geralmente da dificuldade em se implementar estratégias planejadas adequadamente [14].

Antes da vacinação quase meio milhão de americanos contraíam o sarampo anualmente [12]. Em 1963, foi aprovada a primeira vacina contra a doença naquele país, levando a uma queda significativa no número de casos em 5 anos (de 458.000 para 22.000 [8]). Um surto em 1977 levou ao início da vacinação compulsória naquele país. Em 1989, foi introduzida a segunda dose da vacina, adicionando maior eficácia no controle da doença. Novo surto em 2005, em Indiana, mostrou a natureza altamente infectante do vírus e a vulnerabilidade de comunidades vacinadas em um mundo onde o vírus continua a circular [12].

Atualmente a OMS preconiza a primeira dose da vacina aos nove meses de vida. Uma "segunda oportunidade" de se vacinar contra o sarampo deveria ser oferecida a todas as crianças entre nove meses e 15 anos de vida – cobrindo os não vacinados e os que eventualmente não tivessem respondido à primeira dose (cerca de 15% dos vacinados aos nove meses) [4].

Uma das principais causas do insucesso vacinal no primeiro ano de vida seria, conforme dito anteriormente, a presença de anticorpos maternos induzidos por infecção prévia ou por vacinação, que passam para o neonato pelo sangue de cordão [133] e que preveniriam a vacinação bem sucedida, mas que poderia não ser capaz de proteger contra a infecção pelo vírus selvagem [2, 7], tornando esse grupo altamente susceptível à doença mesmo em comunidades com alta cobertura vacinal [12]. Aparentemente a imunidade transferida pelas mães tem diminuído com o passar dos anos, uma vez que os anticorpos transferidos são na maior parte das vezes aqueles produzidos no segundo ano de vida, após a segunda vacinação [12]. Esse fato se torna ainda mais significativo se levarmos em conta a maior gravidade do sarampo na população de lactentes jovens.

Cerca de 5% dos vacinados geralmente não desenvolvem anticorpos contra o sarampo[8], sendo que a imunidade em adultos jovens poderia eventualmente diminuir com o tempo, tornando-os progressivamente susceptíveis ao vírus selvagem [8, 12]. Outra preocupação existente quanto à imunogenicidade se refere ao fato do vírus atenuado do sarampo ser atualmente administrado juntamente aos vírus atenuados da caxumba e da

rubéola. A administração dos três vírus concomitantemente poderia, em princípio, resultar em menor soroconversão ou maior incidência de efeitos colaterais que as obtidas com o uso dessas vacinas isoladamente. Na prática clínica, entretanto, não se notou diferença entre o uso isolado da vacina do sarampo e o seu uso em conjunto com os outros vírus atenuados [151].

A linhagem viral Edmonston, da qual a maior parte das vacinas se derivou, foi inicialmente isolada em 1954 por Enders. Esse vírus foi posteriormente submetido a múltiplas passagens em células amnióticas e de rim humano, adaptado a ovos e submetido a múltiplas passagens em células embrionárias de galinha para produzir a primeira linhagem vacinal, licenciada em 1963. Essa linhagem causava febre e *rash* em 30-60% dos lactentes, sendo posteriormente substituída por linhagens mais atenuadas [135].

Atualmente a linhagem vacinal em uso no Brasil é a Schwarz. Seu seqüenciamento levou à conclusão que essa linhagem era semelhante à outra linhagem vacinal – a Moraten [30]. Estudo comparativo entre essas linhagens e a Edmonston levou à conclusão que todas apresentavam altas taxas de seroconversão – 98%. De forma interessante, a linhagem Schwarz causou mais febre entre os vacinados que a Moraten (diferença estatisticamente significativa). A linhagem Edmoston, por outro lado, não somente causou mais febre que as demais, mas também maior incidência de *rash* cutâneo [3].

Apesar de todas as vacinas disponíveis serem produzidas com vírus do genótipo A, o soro de pessoas vacinadas foi capaz de neutralizar todos os genótipos virais (com eficiências variadas) [28]. A vacinação foi bem sucedida em proteger contra a infecção pelo vírus selvagem, entretanto vários foram os efeitos colaterais associados à mesma. A imunossupressão observada após a doença pôde, portanto, ser detectada após a vacinação [135], mas com menor intensidade [85]. Vacinas com altos títulos, no entanto, levaram a aumento na mortalidade em meninas [152], aumento esse provavelmente decorrente desta imunossupressão [8]. 5% dos vacinados podem também apresentar *rash* cutâneo e febre [28]. O período crítico para o surgimento da febre seria ente o quinto e o décimo segundo dias após a vacinação [3], com duração média de um a dois dias [153]. Conjuntivite pós vacinação foi descrita em cerca de 2% dos casos [153].

Reações anafiláticas foram descritas, com incidência entre 1:20.000 a 1:1.000.000. O uso de gelatina modificada, ao invés das proteínas do ovo, seria o principal responsável

pela maior parte dos episódios de anafilaxia após a vacinação [153]. Desta forma, acredita-se hoje que seja seguro o uso da vacina contra o sarampo mesmo em pacientes com relato de anafilaxia prévia com o uso de ovo [153].

Do ponto de vista neurológico foram descritos casos de encefalomielite pós vacinação e de crises convulsivas febris, sendo sugerida profilaxia com anti-térmicos no caso da sua ocorrência [153]. Apesar de infreqüentes e controversas, outras doenças tem sido associadas à vacinação pelo sarampo, como o autismo [154]. A associação com MMR e autismo, por outro lado, não resistiu a estudos mais cuidadosos [155,156].

Também já foi relatada associação entre a vacinação com MMR (vacina contra sarampo, caxumba e rubéola) e a púrpura trombocitopênica idiopática [153].

Em pacientes portadores de imunodeficiência celular ou combinada o uso da vacina contra o sarampo deve ser evitado. Monafo e cols, por exemplo, descreveram caso de criança portadora de imunodeficiência combinada grave que evoluiu para o óbito devido a sarampo disseminado secundário à vacinação. O vírus foi encontrado no fígado, pulmão, baço, linfonodos e na adrenal [157]. Foram também relatados, após a vacinação, 3 casos de encefalite por corpo de inclusão levando a óbito pacientes com imunodeficiência celular e um caso de pneumonia de células gigantes em adulto portador de AIDS [153].

1.9 Considerações finais

Apesar da queda progressiva do número de casos de sarampo notada mundialmente, mais de 200.000 pessoas ainda morrem em decorrência do vírus anualmente [4], ocorrendo surtos esporádicos mesmo em países que já possuem um controle eficiente da doença, como o Brasil [11]. Desta forma, estudos que visem ao desenvolvimento de vacinas mais eficientes (para serem usadas principalmente no primeiro ano de vida, quando as vacinas atuais ainda se mostram ineficientes) e a uma melhor compreensão dos mecanismos envolvidos na patogênese viral ainda se fazem necessários.

Há mais de uma década, o uso de genética reversa vem sendo aplicado em experimentos envolvendo o vírus do sarampo [158, 159]. Seu uso tem auxiliado não somente na compreensão do papel das proteínas virais na patogênese da doença, mas tem também trazido esperança do uso do vírus do sarampo como um agente terapêutico, servindo como um vetor em terapias genéticas. Vírus recombinantes que podem entrar em

células que expressam seletivamente marcadores tumorais foram desenvolvidos e direcionados para a lise de células alvo, sendo o vírus do sarampo um dos potenciais candidatos para tais terapias [160, 161]. Em ratos transgênicos susceptíveis ao sarampo (que expressam o receptor CD46) foi descrita lise tumoral e melhora clínica significativas com uso de vírus atenuado com tropismo para o receptor do folato, desenvolvido para ter como alvo células de câncer de ovário [162]. Vírus derivado da cepa vacinal Edmonston direcionado a infectar células ativadas do endotélio vascular também foi capaz de induzir a regressão de mieloma múltiplo resistente em ratos portadores de imunodeficiência combinada grave [163].

O uso das proteínas do vírus como agentes terapêuticos poderia também ser de grande utilidade, uma vez que foi descrita melhora dos sintomas da dermatite atópica e da síndrome nefrótica durante a infecção aguda pelo sarampo [108]. Em ratos deficientes da apolipoproteína E, que apresentam lesões ateroscleróticas com 30 semanas de vida, o uso da nucleoproteína do vírus promoveu uma resposta antiinflamatória das células T regulatórias do tipo I, com aumento na produção de IL-10 e inibiu a acumulação de macrófagos e de células T nas lesões. Houve, consequentemente, redução significativa do desenvolvimento de novas placas ateroscleróticas e inibição da progressão das lesões estabelecidas [164].

Por fim, o porquê das linhagens vacinais serem menos patogênicas que as selvagens ainda hoje não foi esclarecido. Estudos comparativos entre essas linhagens seriam úteis não somente para uma melhor compreensão dos mecanismos envolvidos na patogênese do vírus selvagem, mas também contribuiriam para o desenvolvimento de vacinas mais eficientes e seguras.

PARTE II

OBJETIVOS

2. OBJETIVOS

Os mecanismos de atenuação do vírus vacinal do sarampo ainda não foram suficientemente caracterizados. Sabemos hoje que a linhagem vacinal consegue penetrar as células através do receptor CD46, mantendo a capacidade de se ligar ao receptor primário das linhagens selvagens – CD150. Desta forma, decidimos comparar em tecido linfóide humano ex-vivo, três linhagens selvagens com uma linhagem vacinal do vírus do sarampo, avaliando eventuais diferenças no tropismo, na capacidade de disseminação e de depleção celular entre as mesmas.

PARTE III

MATERIAL E MÉTODOS

3. MATERIAL E MÉTODOS

3.1 Linhagens celulares e virais

Células Vero/hSLAM (células de rim de macaco verde africano expressando SLAM humano [47]) foram gentilmente fornecidas pelo Dr. Y. Yanagi (Universidade de Kyushu, Fukuoka, Japão) e eram mantidas a 37°C, em incubadora de CO_2 a 5%, em meio de Eagle modificado por Dulbecco (DMEM) com 10% de soro bovino fetal (FCS), na presença dos antibióticos G418, penicilina e estreptomicina em uma concentração de 0,5 mg/ml.

Para resgate viral, foram utilizadas células auxiliares 293-3-46 (células derivadas de rim humano embrionário e que expressam de forma estável as proteínas virais N, P e também a polimerase T7), gentilmente cedidas por Dr. D. Gerlier (Universidade de Lion, Lion, França [158]). As culturas foram mantidas a 37°C em incubadora de CO_2 a 5%, em meio de Eagle modificado por Dulbecco (DMEM) com 10% de soro bovino fetal (FCS) e na presença de G418 na concentração de 1,2 mg/ml.

Células B95a (células linfoblastóides de sagüi) e as células Vero foram cultivadas em DMEM sem aditivos.

As linhagens virais selvagens MVwtD4 e MVwtD8 (vírus selvagens do sarampo dos genótipos D4 e D8, respectivamente) foram fornecidas por P. Rota (Centro para Controle e Prevenção de Doenças, CDC, Atlanta, Geórgia; ver referência 23 para revisão). Para propagação viral, foram empregadas 3 passagens em células Vero/hSLAM.

Os vírus recombinantes MVwtICB, fornecidos por Dr. K. Takeuchi (Universidade de Tsukuba, Tsukuba, Japão [28]) e a linhagem vacinal MVvac [119] foram gerados a partir de DNA complementar com cópias completas dos genes ("*full lenght*" cDNA) como descrito por Radecke et al. [158]. Sincícios foram isolados e propagados em células Vero/hSLAM e posteriormente amplificados.

3.2 Preparação do estoque viral

Os estoques virais foram preparados a partir de células Vero/hSLAM infectadas com uma multiplicidade de infecção (razão entre partículas infecciosas virais sobre o número de células alvo – MOI) de 0.03 $TCID_{50}$/célula ($TCID_{50}$= dose necessária para infectar 50% de uma cultura de tecido) com cada linhagem viral e incubadas a 32°C. As

células foram mecanicamente descoladas do frasco de cultura contendo meio Opti-MEM (Invitrogen) e as partículas virais liberadas através de 2 ciclos de congelamento/descongelamento. Os títulos virais foram determinados por $TCID_{50}$ em células Vero/hSLAM de acordo com o método Spearman-Kärber [165].

3.3 Avaliação do tropismo da linhagem vacinal e das linhagens selvagens

Células B95a, que expressam apenas SLAM ($5x10^5$ células por poço), células Vero, que expressam apenas CD46, e células Vero/hSLAM, que expressam tanto SLAM como CD46 ($2.5x10^5$ células por poço), foram cultivadas em placas de cultura de poliestireno de 12 poços e posteriormente infectadas com os vírus MVvac, MVwtICB, MVwtD4, e MVwtD8 (com 0.1 $TCID_{50}$ /célula, diluídas em 0,5ml de meio Opti-MEM). Após duas horas de cultura a 37°C, o meio de cultura Opti-MEM foi substituído por meio DMEM contendo 10% de FCS. Após 36 horas de infecção, a cultura foi analisada em microscópio de contraste e a formação de sincício foi observada e registrada através de fotografia.

3.4 Manutenção e infecção do tecido linfóide

Amígdalas humanas removidas durante amigdalectomias de rotina foram dissecadas em pequenos blocos de aproximadamente $2mm^3$ que eram colocados sobre esponjas de gelatina (*gelfoam*) em grupos de nove blocos. Cada fragmento de gelfoam (que consistia em ¼ de seu tamanho comercial) foi embebido em meio de cultura (RPMI associado a 1% de piruvato de sódio, 1% de aminoácidos não essenciais, 1% de solução de anfotericina B a 250μg/ml, 0,1% de solução de gentamicina a 50μg/ml e 15% de FBS) e colocado em cada uma das cavidades de uma placa de cultura de poliestireno de 6 poços, na presença de 3ml de meio de cultura. As placas foram mantidas em cultura em incubadora com 5% de CO_2 a 37°C.

Para cada condição experimental foi usada uma placa de 6 poços, contendo um total de 54 blocos de tecido (9 blocos sobre cada gelfoam / 6 poços). Cada bloco de $2mm^3$ continha em média 3.5×10^6 células (3.5×10^5 linfócitos). Os experimentos foram repetidos usando tecidos de vários doadores.

Para a infecção, cada bloco de tecido foi inoculado com 5μl de uma solução viral que continha 500 $TCID_{50}$/ml, com a exceção de dois experimentos cujos blocos de tecido

foram inoculados com uma solução de 170 $TCID_{50}$/ml da linhagem viral MVwtD4. O meio de cultura foi trocado a cada 3 dias. Culturas contendo blocos de tecido não infectados foram usadas como controle.

3.5 Obtenção de suspensão celular

Experimentos iniciais de cinética foram realizados nos dias 3, 6, 9 e 12 após infecção para determinação do dia com maior percentual de células infectadas. Uma vez evidenciado que tal pico ocorria entre os dias 6 e 9, optou-se por manter a análise das amostras apenas no dia 9.

Desta forma, no dia 9 após infecção os blocos de tecido de cada condição experimental foram recolhidos e colocados em um tubo Eppendorf contendo 500 μL de meio de cultura e 500 μL de solução de colagenase IV a 1%. Após incubação por 30 minutos em agitação a 1.000 rpm, em placa aquecida, os blocos foram rompidos com um pequeno pistilo plástico para dispersão das células. Em seguida, a suspensão obtida foi filtrada em tela de nylon de 40 μm. A solução de células foi então lavada com uma solução salina tamponada com fosfato (PBS), centrifugada e o sedimento obtido ressuspenso em PBS. A viabilidade celular foi verificada através do sistema LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen).

3.6 Ensaios de citometria de fluxo

Foram utilizados dois protocolos para análise fenotípica das suspensões celulares. O primeiro protocolo de marcação consistiu na determinação da eficiência de infecção de células T virgens e de memória. Para tanto, as células foram incubadas por 30 minutos com diferentes anticorpos. A quantidade de anticorpos usados foi definida por titulação prévia. Nos primeiros 5 experimentos se utilizou a seguinte combinação: anti-CD3 Cy7-ficoeritrina (PE), anti-CD4 Cy5.5-aloficocianina (APC), anti-CD8 *pacific blue*, anti-CD62L Cy7-APC, anti-CD45RA Cy5.5 PE e anti-CD45RO Cy.5 PE (Invitrogen-Caltag) e anti-CD150 PE (BD Pharmingen). Nos últimos 4 experimentos utilizou-se a seguinte combinação de anticorpos: anti-CD3 Cy5.5-APC, anti-CD4 610-PE, anti-CD8 *Pacific Blue*, anti-CD19 Cy7-PE, anti-CD62L Cy7-APC, anti-CD45RA *TRI-COLOR*, anti-CD45RO APC e anti-CD150 PE.

Após incubação, realizada em tubos de 5ml de poliestireno, as células foram lavadas e permeabilizadas com o reagente Fix&Perm (Invitrogen-Caltag). Em seguida, utilizou-se anticorpo monoclonal anti-nucleoproteína viral marcado com isotiocianato de fluoresceína (FITC) (Chemicon International) para a identificação das células infectadas pelo vírus do sarampo. As células eram então novamente lavadas com PBS e fixadas com formaldeído a 4%.

Para a avaliação da infecção de células B, células NK e células dendríticas utilizou-se nos primeiros 5 experimentos a seguinte combinação de anticorpos: anti-CD3 Cy7-PE, anti-CD16 e anti-CD56 *Texas Red*-PE, anti-CD14 APC, anti-CD19 Alexa 750-APC, anti-HLA-DR Cy5.5 PE (todos produzidos por Invitrogen-Caltag) e anti-CD150 PE (BD Pharmingen). Nos 4 últimos experimentos optou-se por realizar-se uma avaliação mais detalhada das células dendríticas, sendo então utilizados: anti-CD3 Cy5.5-APC, anti-*CD16 e* anti-*CD56 Texas Red*-PE, anti-CD14 Alexa 750-APC, anti-CD19 Cy7-PE, anti-HLA-DR Cy5.5-PE e anti-CD13 *TRI-COLOR* (todos produzidos por Invitrogen-Caltag) e anti-CD20 Cy7-PE, anti-CD11c APC e anti-CD123 PE (produzidos por BD Pharmingen). Em todos os nove experimentos foi também realizada a permeabilização das células e marcação intracelular da nucleoproteína (conforme descrito anteriormente).

Os parâmetros fenotípicos e morfométricos das células foram analisados em citômetro de fluxo LSR II, equipado com lasers de 355, 488, 532, 407 e 638 nm e software DIVA 4.1.2. O programa FlowJo versão 8.3 (Tree Star, Ashland, OR) foi utilizado para a análise dos resultados.

3.7 Estimativa da depleção celular

Para estimar a depleção celular, foram comparados os tecidos infectados com controles não infectados. Microesferas (Invitrogen-Caltag *counting beads*) foram adicionadas anteriormente à aquisição e o número de células foi estimado de acordo com as instruções do fabricante. Os resultados foram normalizados pelo peso do tecido, utilizando-se a seguinte fórmula: total de células/número de esferas de contagem/peso.

3.8 Análise estatística

Tanto os níveis de infecção viral quanto as proporções dos vários subtipos de leucócitos variaram entre os experimentos [166, 167]. Para cada experimento foram comparados os tecidos infectados com os controle não infectados, obtidos de "doadores" individuais, em réplicas de 54 blocos. Para analisar a depleção celular nos tecidos infectados realizou-se a normalização dos dados como percentual do tecido controle. A análise estatística consistiu no cálculo da média, desvio padrão, erro padrão e valores de P utilizando o teste t de Student. Considerou-se como significativo valor de $p \leq 0,05$.

PARTE IV

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4. REFERÊNCIAS

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PARTE - V

ARTIGO

MAJOR ARTICLE

Measles Virus Vaccine Attenuation: Suboptimal Infection of Lymphatic Tissue and Tropism Alteration

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The mechanisms of measles virus (MV) vaccine attenuation are insufficiently characterized. Because the Edmonston vaccine strain can enter cells through CD46 in addition to the primary MV receptor signaling lymphocyte activation molecule (SLAM or CD150), we asked whether and how its tropism is altered. In human tonsillar tissue, this vaccine strain infects naive ($CD45RA^+CD62L^+$) T lymphocytes, which express SLAM very infrequently, with much higher efficiency than do wild-type strains. By contrast, it infects B lymphocytes, macrophages, and NK cells with significantly lower efficiencies than those of wild-type strains. Infection levels by wild-type strains correlate with the frequency of SLAM expression and are highest in B cells, which are 40%–55% infected. SLAM-expressing T cells are more readily infected by all MV strains than are SLAM-expressing B cells. Thus, vaccine attenuation may be caused by tropism alteration in combination with suboptimal replication.

Measles virus (MV) infection causes >600,000 deaths yearly because of severe immunosuppression facilitating secondary infections [1, 2]. The live attenuated MV vaccine strain protects against measles and is only moderately immunosuppressive, but the mechanisms underlying its attenuation have been insufficiently studied. Many mechanisms of MV-induced immune suppression have been considered, but there is no consensus about the types of immune cells sustaining viral spread [3, 4]. Moreover, the magnitude of infection during the early phases of acute measles has been underestimated, because clinical samples often contain very few or no virus-infected cells. However, MV can be isolated frequently from both respiratory and urine samples obtained very close to rash onset.

In vivo studies of MV virulence are limited to a few experimental systems—macaques [5, 6] and genetically modified mice [7, 8]—but the disease is faithfully reproduced only in the costly macaque model. Therefore, infections of natural hosts by the morbilliviruses canine distemper and rinderpest have been characterized [9, 10]. These viruses spread initially in lymphatic organs, then in epithelia [11]. Infection of peripheral blood mononuclear cells (PBMCs) follows that of lymphatic organs and is less extensive. The few data available from analyses of early MV spread in macaques are consistent with strong replication in lymphatic organs preceding limited PBMC infection [6, 12].

Studies of MV tropism and of its immunosuppression mechanisms have been based on activated human PBMCs or cell populations derived therefrom. MV infects T and B lymphocytes, monocytes [13, 14], and monocyte-derived dendritic cells (DCs) [15–18] at low levels, but virus production is, at best, inefficient. Therefore, we studied MV tropism in a more adequate system based on human lymphoid tissue cultured on collagen sponge gel [19, 20]. This system, which was originally developed for the study of HIV pathogenesis

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[21], has been used to characterize the mechanisms of interference between MV and HIV infections using a vaccine lineage strain and a wild-type–derived laboratory-passaged Chicago-1 strain [22].

Here, we used the system to compare the spread of a bona fide MV vaccine strain with that of 3 bona fide wild-type strains. Two of the wild-types were recently isolated in the United States [23] and propagated exclusively on Vero/human signaling lymphocyte activation molecule (hSLAM or CD150) cells [24]. The third was derived from infectious MV cDNA [12] and is amenable to reverse genetics. We also used a virus from an infectious cDNA derived from a vial of the Moraten vaccine strain [25]. Moraten is one of several vaccine strains originating from the clinical isolate Edmonston, differs by 1–6 aa/protein from the lowest available passage stock of this isolate, and is identical to the Schwartz vaccine strain, despite a nominally different origin [26].

We reasoned that the cellular tropism of the Moraten/Schwartz vaccine strain may differs from that of wild-type strains. These strains use as a receptor SLAM, a T cell activation molecule [27] also expressed in certain B lymphocytes [28] and other activated lymphoid cells [24, 29, 30]. This vaccine strain can also enter cells through the ubiquitous regulator of complement activation CD46 [31–33]. By comparing the tropism of this vaccine strain with that of wild-type strains, we noticed that it infects less efficiently every cell type that we examined, except for T lymphocytes. Because naive T lymphocytes infrequently express SLAM, we asked whether the vaccine strain infects these cells more efficiently than do the wild-type strains. It did, adding tropism alteration to reduced replication efficiency as a possible cause of immunosuppression.

MATERIALS AND METHODS

Cells and viruses. All cells were cultured and maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) that contained 10% fetal bovine serum. Vero/hSLAM cells (African green monkey kidney cells expressing human SLAM [24], provided by Dr. Y. Yanagi, Kyushu University, Fukuoka, Japan) were cultured in the presence of 0.5 mg/mL G418, penicillin, and streptomycin. Helper 293-3-46 cells for virus rescue [34] were grown in the presence of 1.2 mg/mL G418. B95a (a marmoset B cell line provided by Dr. D. Gerlier, University of Lyon, Lyon, France) and Vero cells were grown in plain medium.

Wild-type strains MVwtD4 and MVwtD8 (provided by P. Rota, Centers for Disease Control and Prevention, Atlanta, GA; see [23] for review) were propagated on Vero/hSLAM for 3 passages. Both recombinant MVwtICB (provided by Dr. K. Takeuchi, University of Tsukuba, Tsukuba, Japan) [35] and MV vaccine strain (MVvac) [25] were generated from full-length cDNA as described by Radecke et al. [34]. Single syncytia were picked and propagated on Vero/hSLAM cells and amplified.

Virus stock preparation. Virus stocks were prepared from Vero/hSLAM cells infected at an MOI of 0.03 $TCID_{50}$/cell with the relevant virus and incubated at 32°C. Cells were scraped in Opti-MEM (Invitrogen), and particles were released by 2 freeze-thaw cycles. Titers were determined by $TCID_{50}$ titration on Vero/hSLAM cells according to the Spearman-Kärber method [36].

Receptor usage of vaccine and wild-type strains. B95a (5×10^5 cells/well), Vero, and Vero/hSLAM (2.5×10^5 cells/well) cells were infected in a 12-well plate with MVvac, MVwtICB, MVwtD4, and MVwtD8 at 0.1 $TCID_{50}$/cell diluted in 0.5 mL of Opti-MEM for 2 h at 37°C. Two hours after infection, the Opti-MEM was replaced with DMEM–10% fetal calf serum. Syncytia formation was photographed under phase-contrast microscopy 36 h after infection.

Tissue maintenance and infection. Human tonsils removed during routine tonsillectomies were dissected, set up in culture, and maintained as described elsewhere [21]. For each experimental condition, 54 tissue blocks were used: each 1–2-mm/side cubic block (3.3 mg) contained ~3.5×10^6 cells (i.e., 3.5×10^5 lymphocytes). Experiments were repeated using tissues from several donors. For MV infection, each block of tissue was inoculated with 5 μL of a 500 $TCID_{50}/\mu$L viral solution, with the exception of tissue blocks from 2 patients for which the inoculum of MVwtD4 was 170 $TCID_{50}/\mu$L. Culture media were sampled, fully changed, and replaced every 3 days. Matched uninfected tissue blocks were used as controls.

Flow-cytometric analysis. At day 3, 6, 9, or 12 after infection, single-cell suspensions were prepared from tissue blocks by digestion with a 1% solution of collagenase IV (Invitrogen) for 30 min, followed by a mechanical dispersion with a pestle. Dead cells were identified using the LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Invitrogen).

Infections of memory and naive T cells and their correlation with the frequencies of SLAM expression were determined using the following staining: anti–CD3-Cy7–phycoerythrin (PE), anti–CD3-Cy5.5–allophycocyanin (APC), anti–CD4-Cy5.5–APC, anti–CD4–Alexa 610–PE, anti–CD8–Pacific Blue, anti–CD19-Cy7–PE, anti–CD62L-Cy7–APC, anti–CD45RA-Cy5.5–PE, anti–CD45RA-Tricolor, anti–CD45RO-Cy5–PE (all from Invitrogen-Caltag), anti–CD45RO-APC, and anti–CD150-PE (both from BD Pharmingen). Cells were permeabilized by Fix&Perm (Invitrogen-Caltag), and MV infection was determined with a fluorescein isothiocyanate–labeled monoclonal antibody to MV nucleocapsid (N) protein (Chemicon International).

Infection of NK cells, B cells, macrophages, and DCs was assessed using the following staining: anti–CD3-Cy7–PE, anti–CD3-CY5.5–APC, anti–CD16–Texas Red–PE, anti–CD56–Texas Red–PE, anti–CD14-Cy5.5–PE, anti–CD14–Alexa 750–APC, anti-CD19–Alexa 750–APC, anti–CD19-Cy7–PE, anti–HLA-

DR–Cy5.5–PE, anti–CD13-Tricolor (all from Caltag), anti–CD150-PE, anti–CD20-Cy7–PE, anti–CD11c-APC, anti–CD123-PE (all from BD Pharmingen), and the anti–MV-N protein as described above. To estimate cell depletion, we compared the infected tissue with the control. Caltag counting beads were used to estimate cell numbers. Results were normalized by tissue weight.

Statistical analysis. Both the levels of viral infection and the proportions of cells in various leukocyte subsets varied from donor to donor [21, 37]. For each experiment, we compared infected and control tissues obtained from an individual donor in replicates of 54 tissue blocks for each data point. To analyze cell depletion, we normalized the data as the percentage of controls. Statistical analysis consisted in the calculation of mean, SD, SE, and *P* values using Student's *t* test. The significance level was set as $P \leqslant .05$, and actual *P* values are indicated for certain series of experiments.

RESULTS

We documented receptor usage of MVvac and the 3 wild-type strains (MVwtICB, MVwtD4, and MVwtD8) selected for the study using cells expressing different receptors. Figure 1 *(top row)* indicates that all 4 MV strains fuse B95a cells, expressing SLAM but not CD46, whereas only MVvac forms syncytia on Vero cells, expressing only CD46 *(second row)*. MVwtICB, MVwtD4, and MVwtD8 form large syncytia on Vero cells expressing human SLAM *(third row)*. Thus, only the vaccine strain efficiently infects cells expressing CD46. To characterize how vaccine and wild-type MV strains interact with different lymphoid cell types, we inoculated tissue blocks, each containing $\sim 3.5 \times 10^6$ cells, with 2500 infectious units (the MOI was 1:1400) of MVvac, MVwtD4, MVwtD8, or MVwtICB. These experiments were repeated with tissues from *n* donors, and *n* is reported in the table and figure describing each experiment. To ensure the consistency of inocula, viruses were expanded and titered on Vero/hSLAM cells expressing both MV receptors, SLAM and CD46 [24].

As illustrated in figure 2, using flow cytometry, we analyzed 5 cell populations: $CD3^+$ (T lymphocytes), $CD19^+$ (B lymphocytes), $CD14^+$ (macrophages), $CD16^+CD56^+$ (NK cells), and, as the fifth category, all other cells (including DCs). A pilot time-course analysis indicated that virus infection peaked at day 9, and titration of released virus indicated that 800–4000 infectious particles/mL accumulated in the 4 mL of medium bathing 9 tissue blocks infected with vaccine or wild-type viruses over the course of 1-, 2-, and 3-day periods (days 8–9, 7–9, or 6–9; data not shown). Thus, in this culture system, as we reported elsewhere [22], viable viral particles are produced, although modestly. Virus titers do not increase linearly with time, which suggests limited stability. Nevertheless, up to 50% of B lymphocytes and 5%–15% of other lymphoid cell populations were infected at day 9 (see below), documenting significant replication of the inoculum that was washed away and diluted at each medium change (initial MOI, 1:1400 cells). Even in cell monolayers, >90% of infectious MV remains cell associated [38, 39]. Slight changes in the relative amounts of infection of different cell populations sometimes occurred during the 9-day incubation period, but no consistent shifts in the

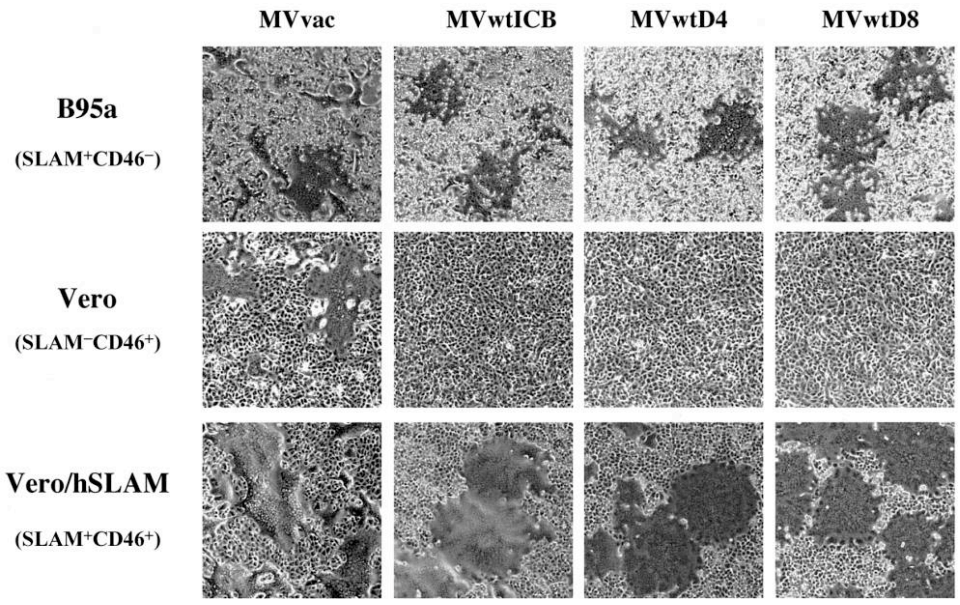

Figure 1. Differential receptor usage by measles virus (MV) vaccine and wild-type strains. Cell types expressing different receptors (indicated at the left of each row) were infected with the viruses indicated at the top of each column and were photographed 36 h later under phase-contrast microscopy to reveal the formation of syncytia. SLAM, signaling lymphocyte activation molecule.

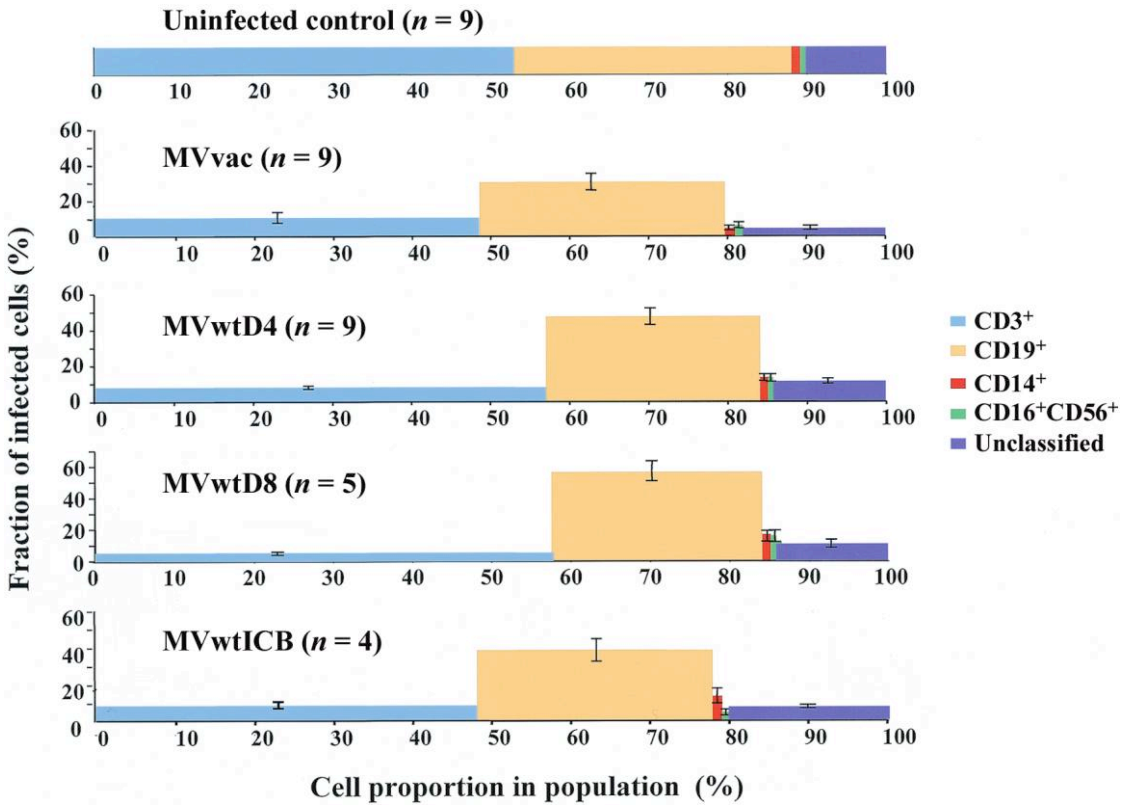

Figure 2. Distribution of cell populations in uninfected and measles virus (MV)–infected tissues, and fractions of infected cells in each population. T lymphocytes ($CD3^+$; *light blue*), B lymphocytes ($CD19^+$; *salmon*), macrophages ($CD14^+$; *red*), NK cells ($CD56^+CD16^+$; *green*), and unclassified cells *(dark blue).* Blocks of human tonsils were left uninfected *(top)* or were infected with 1 of the 4 MV strains indicated (from top to bottom, MVvac, MVwtD4, MVwtD8, and MVwtICB). The no. of donors used with each virus is indicated in each panel. The width of each box corresponds to the average percentage of each cell population. The height of each box corresponds to the average no. of infected cells, expressed as a percentage of that population. Error bars indicate SEs.

relative infection of these cell populations was noticed over time (data not shown). We focused our study on the peak infection levels. Results are illustrated in figure 2 for the vaccine (MVvac, *second panel*) and the wild-type strains (MVwtD4, *third panel;* MVwtD8, *fourth panel;* and MVwtICB, *fifth panel*).

High infection levels in B lymphocytes and intermediate infection levels in macrophages and NK cells. MVwtD4, MVwtD8, and MVwtICB infected ~40%–55% of B cells (mean ± SE, 47.3% ± 4.7%, 56.9% ± 6.3%, and 38.0% ± 6.1%; figure 2, $CD19^+$ cells, *third, fourth,* and *fifth panels,* respectively). The percentage of B lymphocytes infected by MVvac was lower (30.4% ± 6.1%; *second panel*). The differences between the percentages of B lymphocytes infected with MVwtD4 and with MVvac and between those infected with MVwtD8 and with MVvac were statistically significant ($P = .01$ and $P = .04$, respectively). The fractions of B cells infected with MVwtICB and MVvac were similar.

MV replicated in all other cell types, although the peak infection levels were not as high as those in B lymphocytes. In macrophages, the 3 wild-type strains replicated ~3 times better than the vaccine strain (figure 2; $CD14^+$ cells): the percentages of infected cells were 13.0% ± 1.8%, 16.8% ± 3.4%, and 13.0% ± 4.1%, compared with 4.5% ± 1.4%, respectively. For macrophages, the differences between the vaccine and the 3 wild-type strains were all significant (MVwtD4, $P = .0001$; MVwtD8, $P = .01$; MVwtICB, $P = .04$).

In NK cells (figure 2; $CD16^+CD56^+$), MVwtD4 and MVwtD8 multiplied more efficiently than the vaccine strain, whereas MVwtICB multiplied at similar levels. The percentages of infected cells were 12.9% ± 2.0%, 15.7% ± 3.5%, and 4.0% ± 1.6% for the 3 wild-type strains and 6.0% ± 1.6% for the vaccine strain ($P = .01$, MVvac vs. MVwtD4; $P = .05$, MVvac vs. MVwtD8; $P > .05$, MVvac vs. MVwtICB)

We also assessed the cytotoxicity of MV in tonsillar tissue. On average, >60% of cells survived in infected blocks, compared with uninfected tissue blocks. The relative numbers of different cell types remaining on day 9 after MV infection are shown by the widths of the boxes in figure 2. These data indicate that B lymphocytes are preferentially depleted, in accordance with their higher infection levels. On average, their percentages

diminished from 35.0% ± 3.9% in uninfected tissues (figure 2, *top panel, salmon*) to 27.0% ± 3.9% or 26.5% ± 3.9% in MVwtD4- or MVwtD8-infected cells (figure 2, *third* and *fourth panels,* respectively). A more-detailed analysis of cell type depletion is presented below.

Differential infection of memory and naive T lymphocytes. Because inefficient proliferation of human T lymphocytes after contagion is a hallmark of MV-induced immunosuppression, which can be elicited in vitro by a small number of MV-infected cells [40], the analysis of infection efficiency in these cells is of particular interest. Moderate to low infection levels were monitored in $CD3^+$ T lymphocytes, but, remarkably, the vaccine strain infected these cells somewhat more efficiently than did the wild-type viruses (figure 2, *light blue;* MVvac 10.5% ± 3.1% vs. 7.8% ± 0.9% for MVwtD4, 5.4% ± 0.9% for MVwtD8, and 8.4% ± 1.9% for MVwtICB, respectively).

Because the vaccine strain infected all other cell types less efficiently than the wild type, we thought that it might replicate better than those in a T cell subset. Given that SLAM is expressed infrequently in naive ($CD45RA^+CD62L^+$) T lymphocytes but is expressed frequently in memory ($CD45RO^+$) T lymphocytes, we compared the levels of MV infection in these cells (table 1). MVvac infected memory and naive T lymphocytes at ratios close to 1 (0.89 ± 0.16), whereas the 3 wild-type strains infected ~5–6 times more memory than naive T cells (ratios of 5.06 ± 0.44, 6.54 ± 0.89, and 6.07 ± 0.99). The differences between the ratios of the wild-type and the vaccine strains were all statistically significant (MVvac vs. MVwtD4, $P < .01$; vs. MVwtD8, $P < .01$; vs. MVwtICB, $P = .02$).

In figure 3 we document data from 2 donors in whom infection was particularly high or low. In both cases, the ratios of memory:naive cells infected with the vaccine strain were close to 1, and those of cells infected with wild-type strains were close to 5. In donor A, who had low infection, MVvac infected ~1.5 times more memory than naive cells (figure 3*A;* 8.3% vs. 5.4% [compare left panels]). In donor B, who had more-efficient infection, the ratio of MVvac-infected memory: naive cells was ~0.7 (figure 3*B;* 16.1% vs. 21.9% [compare left panels]). In donor A, the wild-type strains MVwtD4 and MVwtICB infected 3–4 times more memory than naive T cells (figure 3*A;* 34.2% vs. 11.7% [compare center panels] and 38.8% vs. 10.7% [compare right panels]). In donor B, MVwtD4 and MVwtICB infected 5–7 times more memory than naive T cells (figure 2*B;* 22.1% vs. 4.4% [compare center panels] and 19.9% vs. 2.8% [compare right panels]). Thus, the MV vaccine strain infects memory T lymphocytes less efficiently but infects naive T lymphocytes more efficiently than do wild-type strains.

Preferential infection and depletion of SLAM-expressing cells. We assessed the extent of the correlation between SLAM expression and MV infection by measuring SLAM expression frequencies in different cell populations in tonsillar tissue blocks. Figure 4 shows that the cell types most efficiently infected by MV are those that most frequently express SLAM: 26% of B lymphocytes, compared with only 6% of macrophages and 4% of NK cells expressed detectable SLAM levels. Among T lymphocytes, only 2.4% of naive cells expressed SLAM, whereas ~19% of memory cells did so. In B and T memory lymphocytes, variability of the frequencies of SLAM expression between tissue blocks from different donors was high, which probably reflects different cellular activation levels.

To assess whether SLAM expression correlates with preferential depletion, we compared cell survival in infected and matched uninfected tissue blocks. Figure 5 presents the relative numbers of cells that survived (mean, median, and SE). Infections with the vaccine or the wild-type MV strains depleted ~1 in 3 tonsil cells: 58%–84% of cells remained alive in infected blocks, compared with uninfected blocks (figure 5*A, gray boxes*). By contrast, depletion of SLAM-expressing cells was more pronounced, and only 23%–35% of these cells survived (figure 5*A, white boxes*). The differences in depletion between the total and the SLAM-expressing cells were statistically significant in MVvac ($P < .01$), MVwtD4 ($P < .01$), and MVwtD8 ($P = .02$). The differences were not significant in MVwtICB ($P = .07$), probably because we tested fewer donors ($n = 4$) in this case.

Preferential depletion of SLAM-expressing cells was most evident in T lymphocytes: 13%–26% of surviving $CD3^+CD150^+$ cells were counted (figure 5*B, white boxes*), compared with 56%–79% of surviving total $CD3^+$ cells (figure 5*B, gray boxes*). By contrast, depletion of SLAM-expressing B cells was less pronounced, 20%–33% of surviving $CD19^+CD150^+$ cells (figure 5*D, white boxes*), even though the depletion of total B lym-

Table 1. Levels of infection of naive and memory T cells.

Virus strain	Infected naive T cells, %	Infected memory T cells, %	Ratio of infected memory to naive T cells
MVvac ($n = 8$)	12.87 ± 4.17	10.12 ± 3.19	0.89 ± 0.16
MVwtD4 ($n = 8$)	4.23 ± 1.01	19.48 ± 2.90	5.06 ± 0.44
MVwtD8 ($n = 4$)	2.61 ± 0.63	16.52 ± 3.66	6.54 ± 0.89
MVwtICB ($n = 4$)	4.20 ± 1.90	21.44 ± 6.09	6.07 ± 0.99

NOTE. Data are mean ± SE no. of cells.

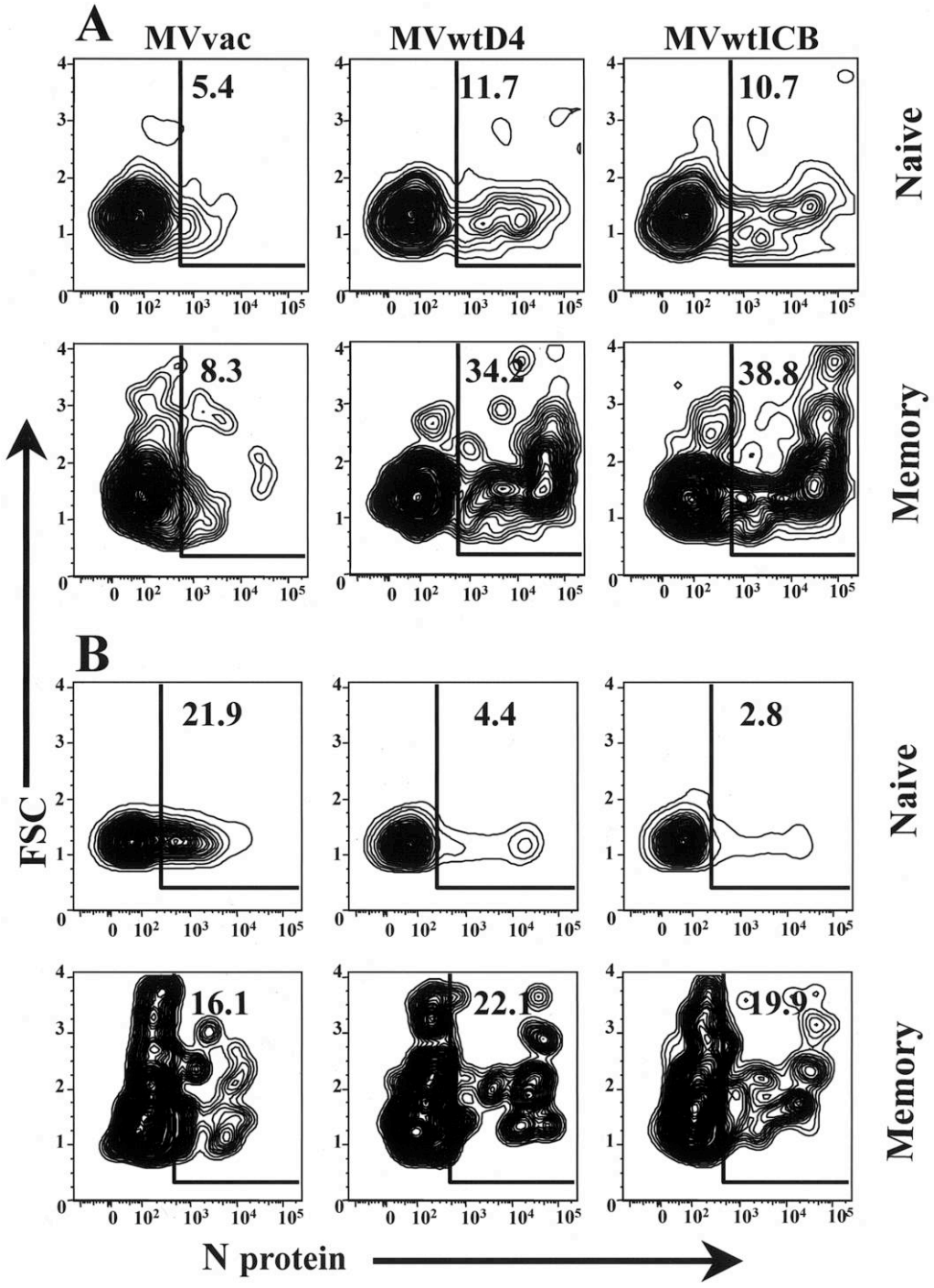

Figure 3. Levels of infection of naive and memory T cells in tissue blocks inoculated with measles virus (MV) strain MVvac, MVwtD4, or MVwtICB. A flow-cytometric analysis of cells isolated from tissue blocks 9 days after infection with MVvac *(left panels),* MVwtD4 *(center panels),* or MVwtICB *(right panels)* is shown. *A,* Results from blocks of tissues from donor A. *B,* Results from blocks of tissues from donor B. Analyses of naive T lymphocytes are shown in the upper row for both patients; analyses of memory T lymphocytes are shown in the lower rows. T cells were gated on the basis of light scatter and CD3 expression; naive cells were then gated on the basis of the coexpression of CD45RA and CD62L and memory cells on the basis of their expression of CD45RO. Contour plots have 2% probability. The bivariate plots show the forward scatter (FSC) vs. nucleocapsid (N) protein expression. The percentage of N-expressing cells in the selected window is indicated in the upper right corner.

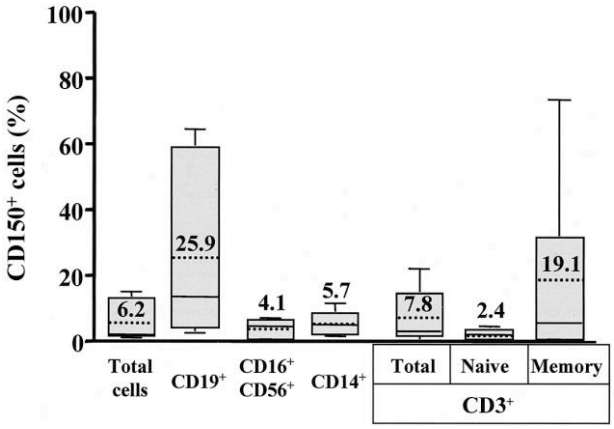

Figure 4. CD150 (signaling lymphocyte activation molecule or SLAM) expression in different cell populations of uninfected tonsillar tissue. In these box-and-whisker plots, boxes extend from the 25th to the 75th percentile, and whiskers indicate the lowest and highest values. The continuous line in the box is the median. The dotted line represents the mean with the corresponding value above it. The no. of experiments done for each population was as follows: total cells, $CD19^+$ cells, and $CD3^+$ cells, $n = 9$; memory and naive $CD3^+$ cells, $n = 8$; $CD16^+CD56^+$ and $CD14^+$ cells, $n = 5$.

phocytes was slightly more pronounced than that of total T lymphocytes (47%–69% of surviving total $CD19^+$ cells; figure 5*C, gray boxes*). Thus, SLAM-expressing T cells are more prone to succumb to MV infection than are SLAM-expressing B cells.

DISCUSSION

Human tonsillar explants as a model for MV infection in lymphoid tissue. We show here that, in human tonsillar tissue, wild-type and vaccine MV replicate robustly, infecting up to 50% of B lymphocytes and 5%–15% of other lymphoid cell populations. Infection results in moderate cell depletion. Predominant B cell infection was documented here in tonsils ex vivo and elsewhere in lymph nodes of macaques on day 7 after inoculation [6].

Infection of tonsillar tissues ex vivo by MV contrasts with HIV-1 infection in this system [21]; HIV-1 infects and depletes $CD4^+$ T cells almost exclusively. However, the numbers of infected cells at any time point are lower than in the case of MV. Nevertheless, in the case of HIV-1 variants entering cells through the ubiquitously expressed CXCR4 protein, >90% of $CD4^+$ T cells are depleted. MV depletes 74%–87% $SLAM^+$ T cells and 67%–80% $SLAM^+$ B cells. For both viruses, ex vivo infection of human tonsillar tissue reflects the respective hallmarks of in vivo infection.

Our data also indicate that the frequencies of SLAM expression in different types of tonsillar lymphoid cells approximately reflect those monitored on the corresponding cell populations circulating in peripheral blood or residing in other lymphoid organs [30]. Thus, tonsillar tissues can be used to identify the cell types supporting MV spread and to gain insights into mechanisms of tissue pathogenesis, even if the fraction of activated cells available in human tonsils surgically removed during routine therapeutic tonsillectomy may exceed that in resting human tissue.

Mechanisms of immunosuppression and vaccine attenuation. The efficiency of infection of different cell populations by the vaccine strain was significantly lower than that of wild-type strains, with one exception: the vaccine strain replicated more

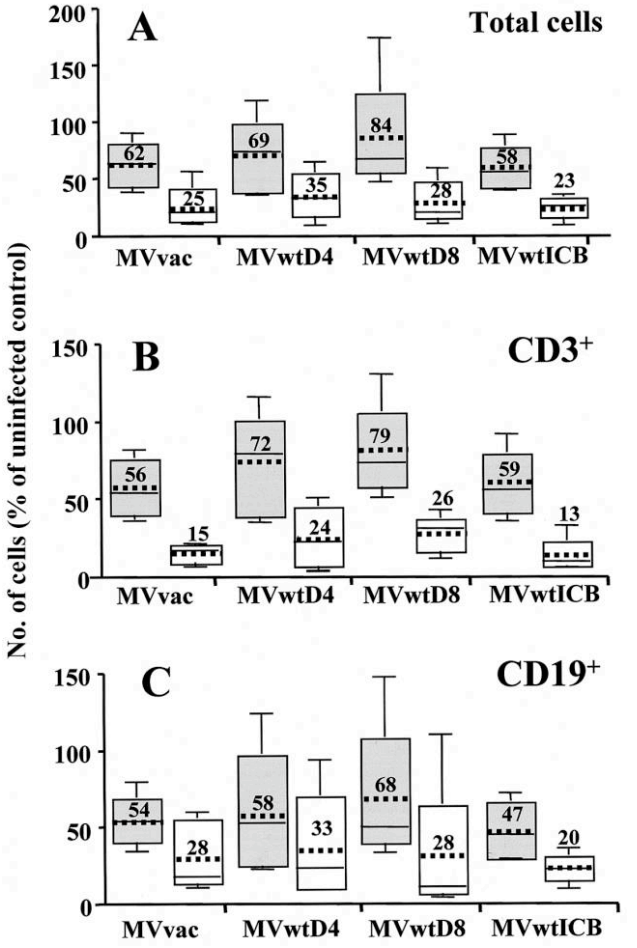

Figure 5. Selective depletion of $CD150^+$ cells 9 days after measles virus (MV) infection. Box-and-whisker plots show the remaining no. of cells, expressed as percentages of control cell populations in uninfected tissue blocks: tonsil cells *(A),* $CD3^+$ cells *(B),* and $CD19^+$ cells *(C).* In each panel, the total no. of cells is indicated with gray boxes and the SLAM-expressing cells with white boxes. Tissues were infected with MV strain MVvac, MVwtD4, MVwtD8, or MVwtICB as indicated on the horizontal axis. Boxes extend from the 25th to the 75th percentile, and whiskers indicate the lowest and highest values. The continuous line near the center of the box is the median. The dotted line represents the mean, with the corresponding value above it. The no. of experiments done for each population was as follows: MVvac and MVwtD4, $n = 9$; MVwtD8, $n = 5$; and MVwtICB, $n = 4$.

efficiently than wild-type strains in naive T lymphocytes. The availability of CD46, the vaccine strain receptor [31, 32] on these cells, probably accounts for the more efficient cell entry. The less-efficient vaccine strain infection of other cell types, such as memory T cells and macrophages, that also express CD46 is puzzling.

We think that, in these cells, vaccine strain infection is very inefficient or disallowed after entry through CD46. Entry of any MV strain through SLAM, a signaling protein, may prime the host cell to support viral replication, whereas entry through CD46 may not. The availability of reverse-genetics systems for the vaccine and a wild-type strain [25, 35] will allow the assessment of whether the capacity to replicate in naive T cells does or does not segregate with the attachment protein, in particular with the residues that interact with CD46 [41]. In this system, the alternative hypothesis that these strains differ in their ability to interfere with host innate immunity can also be addressed. It is also possible that subcutaneous inoculation of the vaccine may account in part for attenuation. This hypothesis can be tested in mice expressing both MV receptors with human-like tissue specificity [8, 42].

MV-induced immune-suppression is a multifactorial process: the number of circulating T lymphocytes is reduced during acute measles, and the CD4:CD8 ratios are often altered [43, 44]. MV also infects tissue macrophages and circulating monocytes, causing their apoptosis [45, 46]. Moreover, DC infection, even if inefficient, may be a factor in immune suppression [4]. Consistent with multiple immune suppression mechanisms, all the immune cells examined in the present study—including lymphocytes, macrophages, NK cells, and DCs—were infected. Wild-type strains infected tissue macrophages responsible for innate response, memory T lymphocytes, and B lymphocytes involved in adaptive immune responses more efficiently than did the vaccine strain. Thus, more-efficient infection of several types of immune cells by wild-type MV strains may contribute to a more-pronounced immune suppression.

SLAM-expressing T lymphocytes succumbed to MV infection more readily than did SLAM-expressing B lymphocytes, which suggests that MV adopts a multiplication strategy compatible with the survival of B lymphocytes residing in lymphatic organs. In animals inoculated with another morbillivirus, canine distemper, primary and secondary lymphoid organs are rapidly infected, initially with little tissue destruction [11]. Thus, the results of MV ex vivo and canine distemper in vivo studies are consistent with a common morbillivirus replication strategy, which was based initially on extensive but moderately cytopathic infection of SLAM-expressing cells, in particular B lymphocytes. Receptor "detargeting" through gain of CD46-dependent entry by the vaccine strain, by altering the viral adaptation to this host cell niche, may elicit an earlier and more-efficient immune response. Combined with suboptimal control of the host innate response, it may result in attenuation.

Acknowledgments

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PARTE - VI

ANEXOS

ANEXO I

OUTROS ARTIGOS PUBLICADOS DURANTE O DOUTORADO - I

HIV-1 pathogenesis differs in rectosigmoid and tonsillar tissues infected *ex vivo* with CCR5- and CXCR4-tropic HIV-1

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Gut-associated lymphoid tissue (GALT) has been identified as the primary target of HIV-1 infection. To investigate why GALT is especially vulnerable to HIV-1, and to determine whether the selective transmission of CCR5-using viral variants (R5) *in vivo* is the result of a greater susceptibility of GALT to this viral variant, we performed comparative studies of CXCR4-using (X4) and R5 HIV-1 infections of human lymphoid (tonsillar) and rectosigmoid tissues *ex vivo* under controlled laboratory conditions. We found that the relative level of R5 replication in rectosigmoid tissue is much greater than in tonsillar tissue. This difference is associated with the expression of the CCR5 co-receptor on approximately 70% of CD4 T cells in rectosigmoid tissue, whereas in tonsillar tissue it is expressed on fewer than 15% of CD4 T cells. Furthermore, tonsillar tissue responds to X4 HIV-1 infection by upregulating the secretion of CC-chemokines, providing a potential CCR5 blockade and further resistance to R5 infection, whereas gut tissue failed to increase such innate immune responses. Our results show that rectosigmoid tissue is more prone than tonsillar lymphoid tissue to R5 HIV-1 infection, primarily because of the high prevalence and availability of R5 cell targets and reduced chemokine blockade. The majority of CD4 T cells express CXCR4, however, and X4 HIV-1 readily replicates in both tissues, suggesting that although the differential expression of co-receptors contributes to the GALT vulnerability to R5 HIV-1, it alone cannot account for the selective R5 infection of the rectal mucosa *in vivo*.

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Keywords: Co-receptor, gut-associated lymphoid tissue, HIV-1, mucosa, tonsil

Introduction

It has been well established that critical events in HIV-1 infection occur in lymphoid tissue [1–8]. More recently, gut-associated lymphoid tissue (GALT) has been identified as the primary target of HIV-1 infection independent of the route of transmission [9–12], probably because gut-associated lymphocytes are predominantly activated memory cells [13]. Typically, infection is transmitted by R5, although both X4 and R5 HIV-1 are often present in seminal fluid [14,15]. To determine whether the selective R5 transmission stems from a greater susceptibility of GALT to this viral variant, we performed comparative studies of X4 and R5 infections of lymphoid (tonsillar) and rectosigmoid tissues *ex vivo* [16].

Here, we show that in rectosigmoid tissue *ex vivo*, R5 virus replicates more efficiently than in tonsillar tissue in association with the expression of the CCR5 co-receptor on the majority of CD4 T cells in rectosigmoid but not in tonsillar tissue. Furthermore, whereas both tissues express significant levels of CXCR4, tonsillar tissue responds to

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X4 HIV-1 infection by upregulating the secretion of CC-chemokines, whereas gut tissue failed to mount such an innate immune response. Rectosigmoid tissue is thus more prone to R5 HIV-1 infection than tonsillar lymphoid tissue; however, both tissues *ex vivo* readily replicate X4 HIV-1. Therefore, the differential expression of co-receptors alone cannot account for the selective R5 transmission across the rectal mucosa *in vivo*.

Materials and methods

Viral stocks

$R5_{SF162}$ and $X4_{LAI.04}$ HIV-1 isolates were obtained through the National Institutes of Health (NIH) AIDS Research and Reference Program and expanded in activated human peripheral blood mononuclear cells to provide the following viral stocks $X4_{LAI.04}$ 166 ng/ml of p24 and 4×10^4 T-cell infectious dose 50% ($TCID_{50}$) per ml; for $R5_{SF162}$ 76 ng/ml of p24 and 10^4 $TCID_{50}$ per ml.

Tissues

Colonic biopsies were obtained at the University of California at Los Angeles (UCLA) from healthy, HIV-1-seronegative individuals recruited from the UCLA Clinical Trial Registry (internal review board approved). Up to 20 biopsies were acquired as previously reported at 30 cm from the anus [16]. Briefly, the biopsies ($8 \times 2 \times 1$ mm) were washed twice in RPMI and mounted on 1 cm^2 gelfoam rafts (Wyeth Pharmaceuticals, Madison, New Jersey, USA) at the medium–air interface in a 24-well tissue culture plate in the presence of 500 μl RPMI plus 10% fetal calf serum supplemented with HEPES (1 mmol; Invitrogen Life Technologies, Carlsbad, California, USA), and a mixture of antibiotics. Each experimental condition was made up of four wells of one biopsy per well; the supernatants from four biopsies per culture were pooled for analysis.

Human tonsils obtained from routine tonsillectomies were dissected into 2 mm blocks and cultured atop gelfoam with a mixture of antibiotics at the medium–air interface as previously described [17,18]. Nine individual blocks were placed on 12×4 mm gelfoam in a well of a six-well plate. Each experimental condition was composed of three wells for a total of 27 blocks whose culture media were pooled. The size of the blocks of both tissues was optimized for culture conditions [16,18].

Infection of explants

Within 5 h of excision, tissue explants were infected by the topical application of 3–6 μl of a stock of $X4_{LAI.04}$ (0.5–1.0 ng of p24 or 120 or 240 $TCID_{50}$) of a clarified viral stock, and in the case of $R5_{SF162}$, by applying 3.0–6.5 μl of a stock of $R5_{SF162}$ (0.25–0.54 ng of p24 or 32–65 $TCID_{50}$). For rectal biopsies, viruses were incubated overnight and washed away to prevent contamination. In the case of tonsillar explants, washing was postponed to day 3 to avoid a massive loss of lymphocytes. The residual inoculum constituted less than 7% of the cumulative p24 production. For both tissues culture medium was collected and replaced every 3 days.

Flow cytometry

Single cell suspensions from rectosigmoid biopsies [19] and from tonsillar tissue [20,21] were prepared as described and subjected to flow cytometry [20,21]. Samples were analysed on a FACSCalibur (BD Biosciences, San Jose, California, USA) using Cellquest software. Lymphocyte numbers were determined by gating on CD45 cells. The absolute numbers of lymphocytes stained for CD45/CD3/CD4, CD45/CD3/CD8, CD45/CD3/CD16/CD56, and CD45/CD3/CD19 with SimulTest (BDIS) were evaluated by Trucount tubes. Cell suspensions of tonsillar tissue were stained with a combination of anti-CD3-Cy7-PE, anti-CD4-Cy5.5-PE, anti-CD8-Tricolor (Caltag, Burlingame, California, USA), anti-CCR5-APC-Cy7, and anti-CXCR4-APC (BDIS); before cell surface staining, counting beads (Caltag) were added to each tube to quantify cell depletion. The samples were acquired on a BD LSRII equipped with the 355, 407, 488, 532 and 638 nm laser lines. Data were acquired with DIVA 4.1.2 (Becton-Dickinson) and analyzed with Flow Jo 6.8 (TreeStar Inc., Ashland, Oregon, USA).

For intracellular p24, cell suspensions from the tissue blocks and biopsies were stained for cell surface markers, were permeabilized with Fix and Perm (Caltag) and stained with 2 μl of the anti-p24 antibody KC57-RD1 or isotype control (Coulter, Miami, Florida, USA).

Cytokine assay (Luminex) of explant supernatants

Macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normally T-cell expressed and secreted (RANTES), stromal-derived factor (SDF)-1α, IFN-γ inducible protein (IP)-10, IFN-γ, tumor necrosis factor (TNF)-α, IL-1β, IL-2, IL-4, IL-10, IL-12, IL-16 and monokine induced by IFN-γ (MIG) were evaluated in culture medium by a multiplexed fluorescent microsphere immunoassay using the Luminex 100 Systems (Luminex, Austin, Texas, USA). Cytokine capture antibodies (R&D Systems, Minneapolis, Minnesota, USA) were coupled to the assay beads. Bead sets coupled with capture antibodies (1250 of each specificity) were mixed with 50 μl standard or culture medium, incubated overnight at 4°C. Bound cytokines were detected with biotinylated antibodies (R&D Systems) and streptavidin–phycoerythrin (Molecular Probes, Eugene, Oregon, USA). Data were analysed with Biorad Bioplex Manager software using a five-parameter fitting algorithm.

Real-time polymerase chain reaction of viral RNA

Viral RNA was extracted from 100 μl culture medium using QiaAmp viral RNA isolation kits [22]. RNA was

eluted in 50 μl water, and 10 μl were reverse transcribed in 50 μl reactions using GeneAmp real-time polymerase chain reaction (PCR) kits (Applied Biosystems, Foster City, California, USA). Primers used for the amplification of viral complementary DNA were designed to amplify segments of the V3–V5 region of the gp120 gene selectively and have been described previously [22]. Real-time PCR assay was performed on the ABI-PRISM 7000 Sequence Detector (Applied Biosystems) using a SYBR Green PCR master mix (Applied Biosystems).

P24 measurement

HIV-1 replication was measured in tissue culture supernatant harvested at days 0, 1, 4, 7, 9 and 12 using the reliance enzyme-linked immunosorbent assay kit (Perkin Elmer, Wellesley, Massachusetts, USA). P24 concentration data were acquired and analysed using delta soft software (Biometallics, Princeton, New Jersey, USA).

Results

R5 and X4 replication in rectosigmoid and tonsillar tissue

The inoculation of rectosigmoid and tonsillar tissue blocks with R5 or X4 variants resulted in productive HIV-1 infection. To account for differences in cellularity between tissue explants, pooled media from 27 tonsillar or four rectosigmoid tissue blocks from each donor were used for each experimental condition, and each experiment was repeated with tissues from a number of donors, denoted below as *n*. The absolute and relative replication of these viral variants differed between the two types of tissues (Fig. 1a,b and Fig. 2a,b).

Replication of $R5_{SF162}$ in rectosigmoid tissue was greater than in tonsillar tissue. When recalculated on a per block basis, an average block of $R5_{SF162}$ HIV-1-infected tonsillar tissue produced between days 3 and

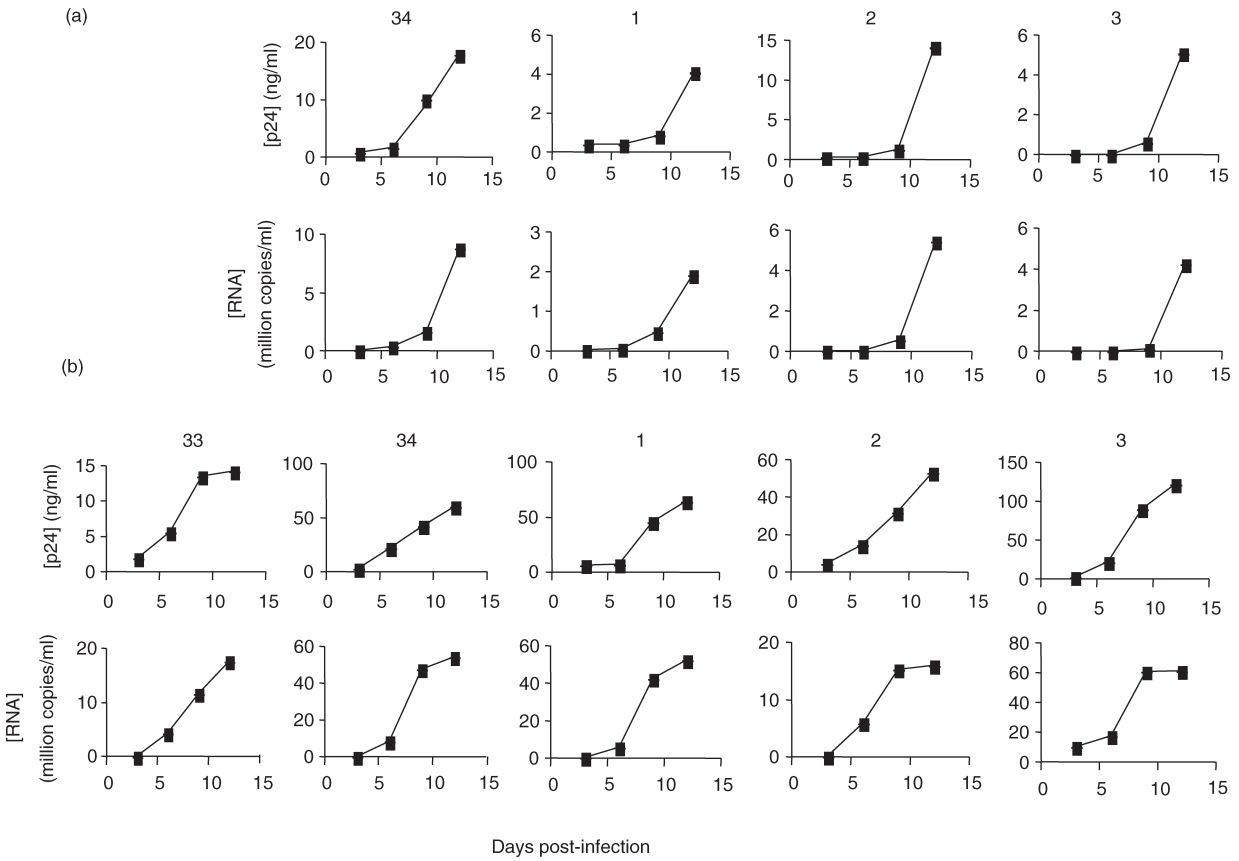

Fig. 1. Replication of X4 and R5 HIV-1 in tonsillar tissues *ex vivo*. Blocks of tonsillar tissues were infected *ex vivo* with (a) $R5_{SF162}$ or (b) $X4_{LAI.04}$. Culture medium bathing the 27 tissue blocks was changed every 3 days and analysed for HIV RNA using real-time polymerase chain reaction and for p24 with enzyme-linked immunosorbent assay. The graphs represent the replication of $X4_{LAI.04}$ in five donors and $R5_{SF162}$ in four donors. The donors are identified by a number on top of each graph. The measurements of viral replication by p24 and by viral RNA were well correlated, as shown by the linear regression analyses of the concentrations of p24 and viral RNA released by tissues infected *ex vivo* with $X4_{LAI.04}$ (c) and $R5_{SF162}$ (d). Cumulative amount of HIV-1 released between days 3 and 12 postinoculation by tonsil explants infected with $X4_{LAI.04}$ and $R5_{SF162}$ measured by p24 (e) and viral RNA (f) (mean ± SEM).

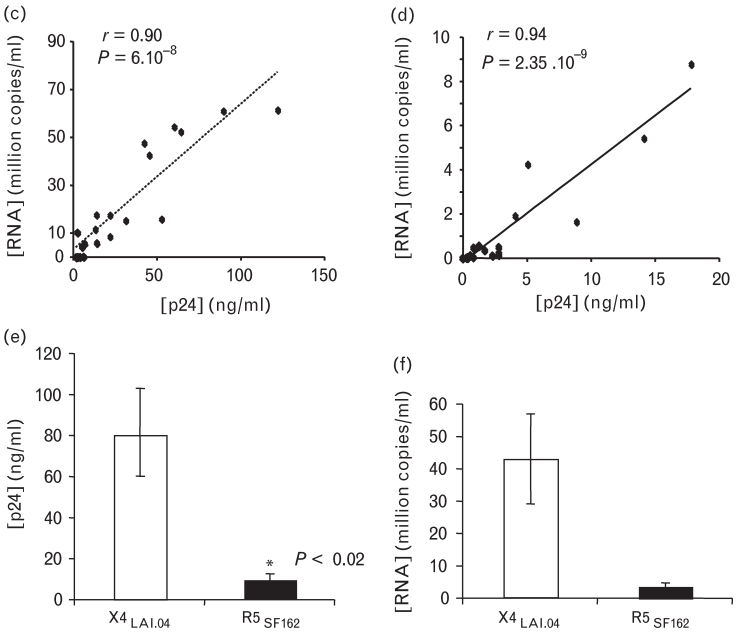

Fig. 1 (*Continued*).

12 postinfection 0.86 ± 0.12 ng of p24, whereas over the same period an average block of rectosigmoid tissue infected with $R5_{SF162}$ produced 4 ± 0.98 ng of p24. Replication of $X4_{LAI.04}$ was greater in tonsil blocks than in rectal biopsies with an average production of 26.7 ± 0.8 and 8 ± 1.53 ng of p24 per block of tonsillar and rectosigmoid tissue, respectively.

We compared R5 and X4 replication in matched blocks of tissues; in tonsils, $R5_{SF162}$ replication was less than $X4_{LAI.04}$ replication. The total accumulation of p24 in supernatants from R5-infected tonsillar tissues reached 12.4 ± 7.6% of that from X4-infected tissues (Fig. 1e) and the total production of viral RNA from R5-infected tissues constituted 15 ± 9% of that in X4-infected tissues (Fig. 1f). In contrast, in rectosigmoid tissue, R5 and X4 replications were similar, with the former reaching 54 ± 17% ($P = 0.3$) of the latter ($n = 5$; Fig. 2e,f). This difference between the HIV-1 replication of R5 and X4 in the two types of tissues was not associated with large differences in replication kinetics [18,23].

There were correlations between the accumulation of p24 and RNA within each tissue type. For tonsillar tissues from each donor, the release of p24 strongly correlated with the release of viral RNA: r varied between 0.92 and 0.99 for $X4_{LAI.04}$ and between 0.95 and 0.999 for $R5_{SF162}$. The correlation between these two parameters remained strong when data points from five different donors were pooled, $r = 0.90$, $P < 0.001$ and $r = 0.94$, $P < 0.001$ for $X4_{LAI.04}$ and R5 isolates, respectively (Fig. 1c,d). For rectosigmoid tissue, the correlation between the production of p24 and viral RNA was not as tight as in tonsils (Fig. 2a,b). This may reflect the larger number of tonsil (27) versus rectosigmoid (four) explants. In individual rectosigmoid experiments, r varied between 0.75 and 0.99 for $X4_{LAI.04}$-infected tissue and between 0.78 and 0.99 for $R5_{SF162}$-infected tissue. For pooled experiments $r = 0.79$, $P < 0.001$ for $X4_{LAI.04}$ and $r = 0.84$, $P < 0.001$ for $R5_{SF162}$, respectively (Fig. 2c,d).

Analysis of the infection of rectosigmoid and tonsillar tissues thus revealed similarities in the kinetics of R5 and X4 HIV-1 replication and a strong correlation between p24 and viral RNA release for both viral variants in both types of tissues. Relative R5 replication was much more efficient in rectosigmoid than in tonsillar tissue.

Lymphocyte subsets in rectosigmoid and tonsillar tissue

To compare HIV-1 infection of rectosigmoid and tonsillar tissues at the cellular level, we phenotyped lymphocytes isolated from tissue blocks [19,24]. The resultant cell suspensions were stained for CD45, CD3, CD4, CD8, CCR5, CXCR4, CD19, CD16, CD56, and p24 and subjected to flow cytometry.

In our analysis of tonsillar cell subsets, we pooled 27 or 54 tissue blocks each containing on average 159 380.3 ± 24 469 T cells, which constituted 56 ± 6% of all tissue lymphocytes. CD4 and CD8 T cells accounted for almost the entire CD3 cell subset, with an average ratio of 4.5 ± 0.4 ($n = 5$). A majority, 66 ± 5%, of CD4 T cells expressed CXCR4 but not CCR5, whereas only 6 ± 2% expressed CCR5 without any apparent expression of CXCR4 and 9 ± 2% expressed both CXCR4 and CCR5 ($n = 15$; Fig. 3a). On day 12 of

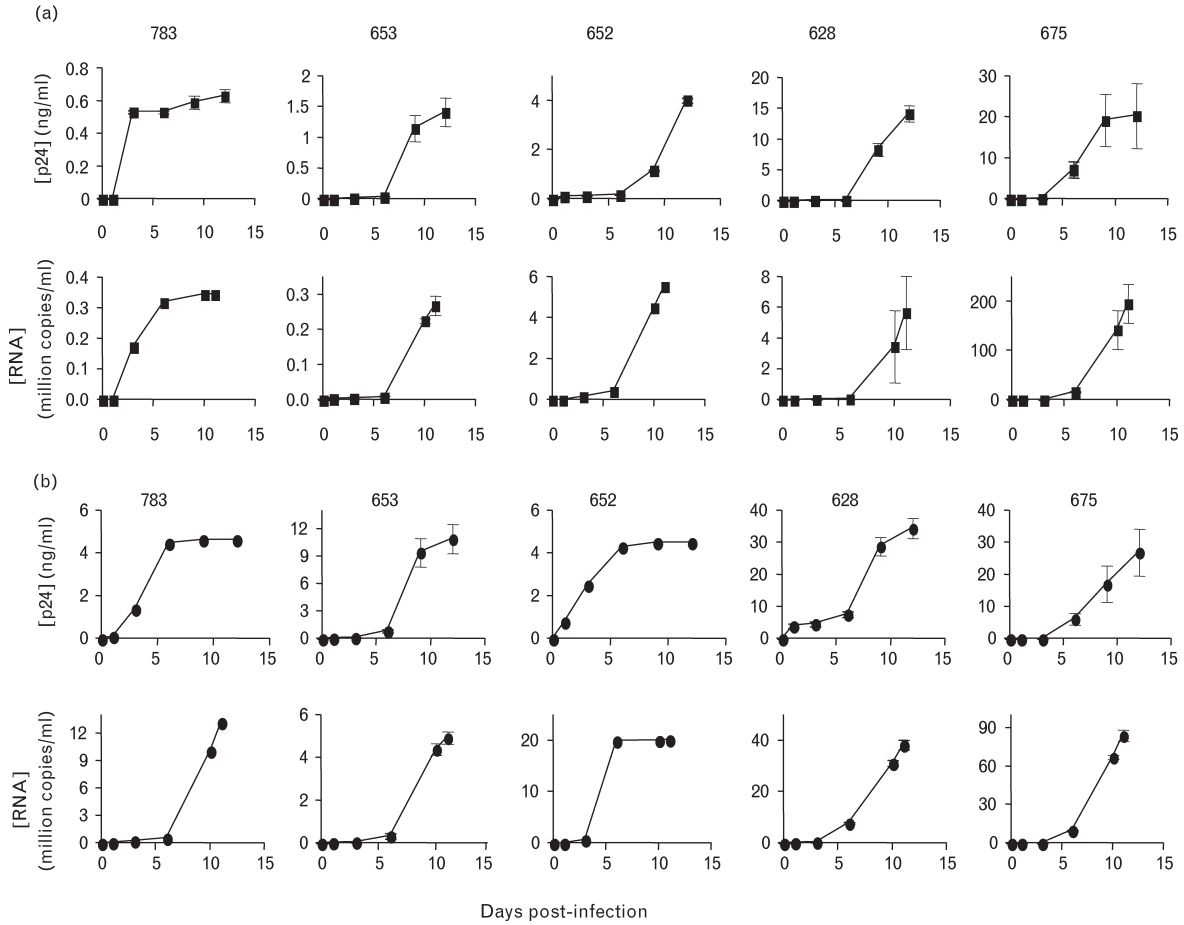

Fig. 2. Replication of X4 and R5 HIV-1 in rectosigmoid tissues *ex vivo*. Rectosigmoid biopsies were infected *ex vivo* with (a) $R5_{SF162}$ or (b) $X4_{LAI.04}$. For each of the five patients and for each experimental condition, four biopsies were cultured and infected by either $X4_{LAI.04}$ or $R5_{SF162}$. The culture medium bathing the four biopsies for each experimental condition was collected, pooled and replaced every 3 days, and analysed for HIV RNA using real-time polymerase chain reaction and for p24 with enzyme-linked immunosorbent assay. The graphs represent the replication of $X4_{LAI.04}$ and $R5_{SF162}$ in five donors. The donors are identified by a number on top of each graph. The measurements of viral replication by p24 and by RNA were well correlated, as shown by the linear regression analyses of cumulative p24 and viral RNA production (over 12 days in culture) in the medium bathing tissues infected *ex vivo* with $X4_{LAI.04}$ (c) and $R5_{SF162}$ (d). Pooled HIV-1 replication data from explants isolated from five donors and infected in set of four for each donor, with $X4_{LAI.04}$ and $R5_{SF162}$ measured by cumulative p24 (e) and viral RNA (f) (mean ± SEM) production over 12 days in culture.

culture, there were no significant changes, with T cells constituting 55.2 ± 4.3% ($n = 5$) of lymphocytes with a CD4 : CD8 ratio of 5.7 ± 1.07 ($n = 5$), and B cells accounting for 29.5 ± 5.7% of lymphocytes ($n = 5$). Further analysis showed that 70 ± 4% of CD4 T cells expressed CXCR4, whereas only 5.4 ± 1% of CD4 T cells exclusively expressed CCR5. Only 3.36 ± 0.6% of CD4 T cells were double positive, whereas the remaining cells were double negative ($n = 19$).

An average block of rectosigmoid tissue contained 367 820 ± 76 394 T cells, which constituted 60 ± 10% of lymphocytes, whereas B cells constituted 32 ± 10% of lymphocytes. CD4 and CD8 T cells accounted for almost the entire CD3 cell subset, with an average CD4 : CD8 ratio of 2.3 ± 0.5 ($n = 5$). HIV-1 co-receptor expression on rectosigmoid CD4 T cells was in agreement with previous reports from our group [21,25,26]; 11 ± 3% of CD4 T cells expressed CXCR4 only, 31 ± 5% selectively expressed CCR5, and 40 ± 8% expressed both CXCR4 and CCR5 (Fig. 3b).

At days 7–10 of culture, T cells made up 86 ± 6% and B cells 11 ± 5% of lymphocytes ($n = 4$). The distribution of T-cell subsets was similar to that observed at day 0, the average CD4 : CD8 ratio remained at the level of 2.54 ± 0.34 ($n = 4$). The expression of chemokine receptors changed slightly from that in freshly isolated tissues and reflected an increase in CXCR4 expression, with 36.3% of CD4 T cells exclusively expressing CXCR4, 47.8% expressing both CXCR4 and CCR5, whereas 12% of CD4 T cells exclusively expressed CCR5.

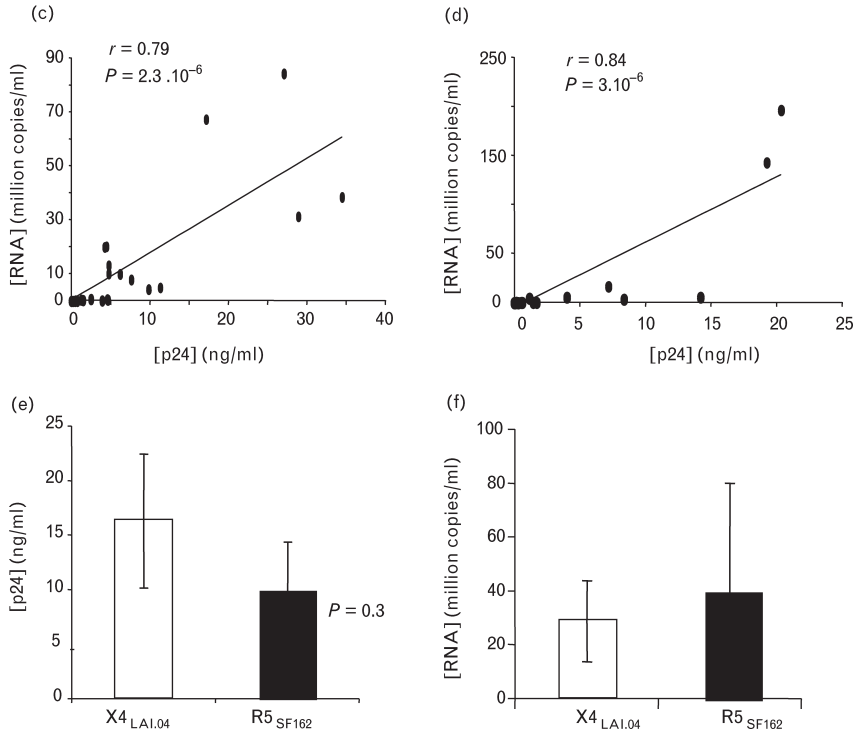

Fig. 2 (*Continued*).

HIV-1 infection of tonsillar and rectal tissue

HIV-1 infection of both tissues resulted in CD4 T-cell depletion. In tonsils, $X4_{LAI.04}$ infection depleted $80 \pm 1\%$ of CD4 T cells ($n = 3$, $P = 0.026$), whereas $R5_{SF162}$ infection depleted $4.57 \pm 4.54\%$ ($n = 3$, $P = 0.9$) of CD4 T cells (Fig. 4a). In rectosigmoid tissue, $X4_{LAI.04}$ infection depleted $79 \pm 6\%$ ($P = 0.02$, $n = 3$) of CD4 T cells, whereas $R5_{SF162}$ depleted $34 \pm 7\%$ ($P = 0.05$, $n = 3$) of these cells (Fig. 4b).

HIV-1-infected CD4 T lymphocytes were identified by intracellular p24 staining. To include CD4 T cells that downregulated CD4 cells because of HIV-1 infection, we analysed $CD3^+CD8^-$ cells because almost all T cells in uninfected tissue express either CD4 or CD8. In tonsillar tissues on day 12 post-$X4_{LAI.04}$ infection, $4.7 \pm 1.7\%$ ($n = 5$, $P = 0.025$) of $CD3^+CD8^-$ cells were p24-positive, whereas in tissues infected with $R5_{SF162}$ the p24-positive fraction constituted $1.22 \pm 0.3\%$ ($n = 5$, $P < 0.01$) of these cells. In $X4_{LAI.04}$-infected rectosigmoid tissue, $10 \pm 5\%$ ($n = 5$) of $CD3^+CD8^-$ lymphocytes were p24-positive, whereas in matched samples infected with $R5_{SF162}$, $3.47 \pm 1.57\%$ ($n = 5$) of these cells were p24 positive.

The frequency of $CCR5^+CD4^+$ T cells in rectosigmoid tissue was approximately fivefold greater than in tonsillar tissue and, accordingly, the frequency of R5 HIV-1

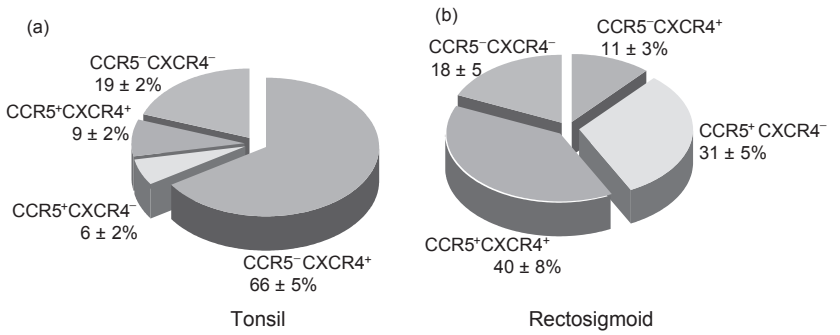

Fig. 3. Expression of HIV-1 co-receptors on CD4 T cells in tonsillar and rectosigmoid tissues. Lymphocytes isolated from tonsillar (a) and rectosigmoid (b) tissues at the time of acquisition were stained for the surface expression of CD3, CD4, CD8, CCR5 and CXCR4 and analysed using flow cytometry. Presented are average distributions of co-receptors on CD4 T cells in tissues from 15 (tonsil) and six (rectosigmoid) donors (mean ± SEM).

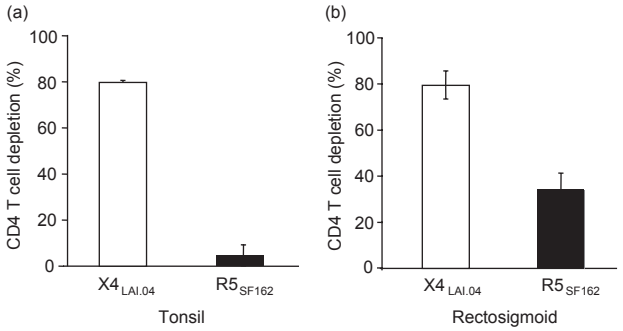

Fig. 4. Depletion of CD4 T lymphocytes in tonsillar and rectosigmoid tissues infected *ex vivo* with HIV-1. Tonsillar (a) and rectosigmoid (b) tissues were infected *ex vivo* with R5 or X4 HIV-1. On day 10–12 post-infection lymphocytes were enumerated using flow cytometry after staining for the surface expression of CD3, CD4, CD8. Depletion was defined as the difference between CD4 : CD8 ratios of uninfected and infected tissues, calculated as $D = (1 - I/C) \times 100\%$, where D is depletion, and C and I are the CD4 : CD8 ratios in control (C) and infected (I) matched tissues. Presented are the average D values (mean ± SEM) for three tonsillar and three rectosigmoid tissues.

productively infected CD4 T cells was greater in rectosigmoid compared with tonsillar tissue.

Cytokines of rectosigmoid and tonsillar tissue

We analysed the modulation of cytokine secretion by HIV-1 infection in rectosigmoid tissues because such a modulation in human tonsils was previously documented [23].

As was shown earlier and confirmed here (Fig. 5), the infection of tonsillar tissue with $X4_{LAI.04}$ increased the secretion of four measured chemokines: from the basal level of 901 ± 120 pg/ml, MIP-1β increased 8.95 ± 1.14-fold ($P < 0.001$), MIP-1α increased 4.30 ± 1.11-fold (basal level 479 ± 70 pg/ml, $P < 0.001$) and RANTES increased 2.5-fold (basal level 1256 ± 437 pg/ml, $n = 5$), although the increase in the latter chemokine was not statistically significant. SDF-1α increased 5.57 ± 0.5-fold (basal level 1295 ± 286 pg/ml, $P < 0.001$). Also, $X4_{LAI.04}$ infection increased the secretion of TNF-α 1.65 ± 0.24-fold (basal level 77 ± 4 pg/ml, $P = 0.024$) and IFN-γ 2.07 ± 0.24-fold (basal level 253 ± 33 pg/ml, $P < 0.001$). There was no increase in IL-1α, IL-1β, IP-10, IL-10 or MIG.

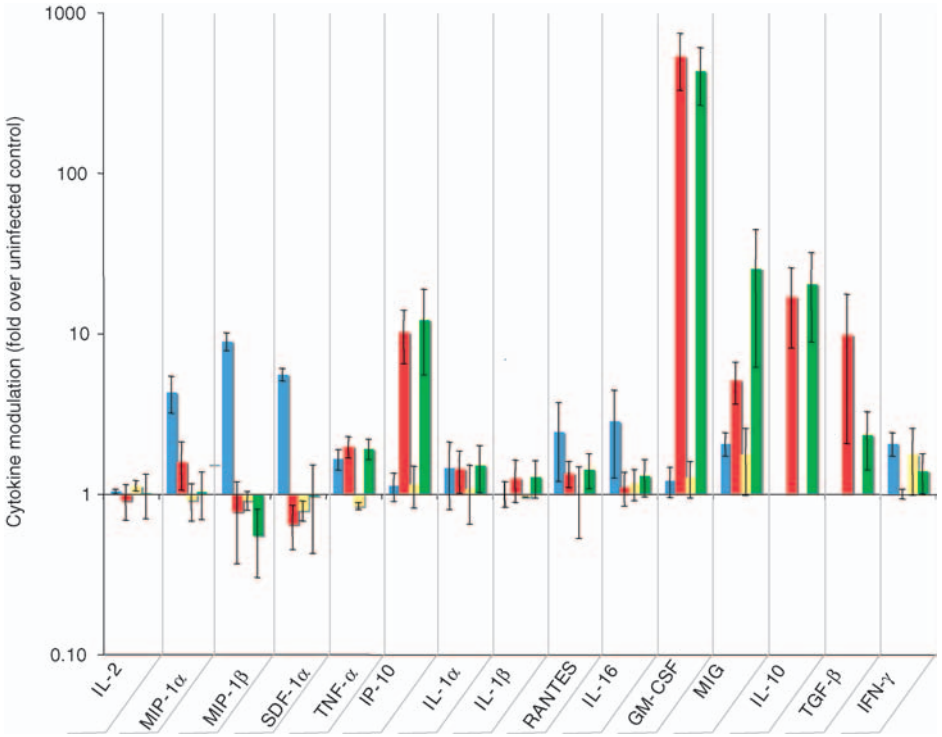

Fig. 5. Chemokine production of tonsillar and rectosigmoid tissues infected *ex vivo* with HIV-1. Tonsillar and rectosigmoid tissues were infected *ex vivo* with R5 or X4 HIV-1 and chemokine concentrations were measured using multiplex bead assay (Luminex) in samples of culture medium collected at days 3, 6, 9 and 12. Presented is a cumulative amount of chemokine released; data points are from tissues of five donors expressed as fold-increase compared with matched uninfected tissues. GM–CSF, Granulocyte–macrophage colony-stimulating factor; IP, IFN-γ inducible protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; RANTES, regulated on activation normally T-cell expressed and secreted; SDF, stromal-derived factor; TGF, transforming growth factor; TNF, tumor necrosis factor. $X4_{LAI.04}$ tonsils; $X4_{LAI.04}$ rectosigmoid; $R5_{SF162}$ tonsils; $R5_{SF162}$ rectosigmoid.

Notably, $R5_{SF162}$ did not change the production of any of the tested chemokines/cytokines.

In rectosigmoid tissue, neither $X4_{LAI.04}$ nor $R5_{SF162}$ modulated the production of chemokines; RANTES, MIP-1α and MIP-1β remained at the basal levels of 1700 ± 167 pg/ml, 7189 ± 900 pg/ml and $25\,553 \pm 5102$ pg/ml, respectively. The production of several cytokines increased upon HIV-1 infection: IP-10 increased 10.21 ± 3.7-fold ($P < 0.001$) in $X4_{LAI.04}$ and 12.22 ± 6.6-fold ($P = 0.003$) in $R5_{SF162}$-infected tissues from a basal level of 5067 ± 2348 pg/ml; MIG increased 5.13 ± 1.5-fold ($P < 0.001$) in $X4_{LAI.04}$-infected tissues and 25.4 ± 19-fold ($P \leq 0.001$) in $R5_{SF162}$-infected tissues from a basal level of 39.6 ± 27.6 ng/ml and IL-10 increased 17 ± 9-fold ($P = 0.003$) in $X4_{LAI.04}$ and 20.39 ± 11-fold in $R5_{SF162}$-infected tissues from a basal level of 1565 ± 884 pg/ml.

To test whether the lack of HIV-1-mediated chemokine induction in rectosigmoid tissue was caused by an inherent incapacity to secrete these chemokines, we activated *ex vivo* rectosigmoid cultures with phytohemaglutinin and measured the secretion of β chemokines. A 3-day phytohemaglutinin activation (10 μg/ml) increased chemokine secretion over the next 7 days: MIP-1β increased 23.1 ± 9.8-fold, MIP-1α increased 11.9 ± 4.4-fold and RANTES increased 16.25 ± 3.56-fold ($n = 3$). The α chemokine SDF-1α was increased 20.5 ± 9.7-fold. Rectosigmoid tissue *ex vivo* is thus capable of chemokine secretion upon stimulation, but in contrast to tonsillar tissue fails to do this in response to HIV-1 infection.

Therefore, in rectosigmoid tissue, neither X4 nor R5 upregulate co-receptor blocking chemokines in contrast to tonsillar tissue, in which X4 infection does. In both types of tissues, however, several immunomodulatory cytokines were upregulated in the course of HIV-1 infection.

Discussion

Whereas both X4 and R5 HIV-1 variants are commonly present in body fluids, R5 HIV-1 is thought to initiate infection selectively, predominantly in lymphoid tissue and dominates its early stages [27]. GALT was recently shown to be the main primary target for HIV-1 at the earliest stages of infection [10–12], not only in cases of anal intercourse but also in other routes of viral transmission. In secondary lymphoid organs, such as lymph nodes or tonsils, infection is established more slowly and viral replication may continue for years [28].

To test whether GALT is particularly vulnerable to R5 HIV-1 infection, we compared infection by R5 and X4 in the tonsils, a secondary lymphoid organ, and in rectosigmoid tissue *ex vivo*. Both human tonsillar and rectosigmoid tissues *ex vivo* support productive infection by R5 and X4 HIV-1 without exogenous stimulation. Another human rectosigmoid explant system was developed earlier by the Dezzutti group for microbicide testing [29], but it requires phytohemaglutinin activation for efficient HIV-1 infection, probably because the semi-polarization of explants in matrigel [29] reduces HIV-1 accessibility to lymphocytes.

Although the absolute levels of HIV-1 replication significantly varied from donor to donor, the relative replication levels of the two viruses in tissues from different donors were similar [30]. This allowed us to pool together data from experiments with different donors.

An average block of rectosigmoid tissue produced more R5 and less X4 HIV-1 than a tonsillar tissue block. On the basis of the CD4 T-cell number, p24 production in tonsils was four to seven times greater than in rectosigmoid tissue for both R5 and X4. The measurement of the relative production of X4 and R5 HIV-1 seems to be more adequate. The fraction of CCR5-expressing cells in rectosigmoid tissue was five times greater than in tonsillar tissue. The majority of these cells in rectosigmoid tissue also expressed CXCR4. The expression of CCR5 by CXCR4-positive CD4 T cells may reflect their constitutive activation in GALT [25]. Also, memory CCR5-positive CD4 T cells are prevalent in rectosigmoid tissue [26]. Therefore, in rectosigmoid tissue, R5 potential targets were much more abundant than in tonsillar tissue. Also, other factors, including a twofold difference in the fraction of CD8 cell anti-HIV factor (CAF)-producing CD8 T cells [31,32] may contribute to the difference in replication in tonsillar and rectosigmoid tissues.

To enumerate the actual R5 and X4 targets we identified them by intracellular p24 staining. The number of productively R5-infected lymphocytes was significantly greater in rectosigmoid than in tonsillar tissue, in agreement with the greater replication of R5 in this tissue. Unfortunately, these data do not provide a basis for the comparison of viral productivity of individual R5 and X4-infected cells in tonsillar and rectosigmoid tissue, because p24 measurement in culture medium represents a cumulative production of virus between media changes, whereas intracellular p24 staining is a snapshot of productive cells that have not yet died from infection.

The relative replication levels of R5 HIV-1 in both tissues correlated with the frequency of CCR5-positive T cells, which in rectosigmoid tissue constituted approximately 70% of the total CD4 T cells at the time of infection, whereas in tonsillar tissue, on average, these cells made up approximately 15% of CD4 T cells. This difference provides the simplest explanation for the difference in the efficiency of R5 HIV-1 infection in these tissues. The abundance of CCR5-positive CD4 T cells in

rectosigmoid tissue makes the cytopathic impact of R5 more pronounced than in tonsillar tissue. In the latter, R5 depletion is barely noticeable, whereas in rectosigmoid tissue more than 34% of CD4 T cells were depleted. These data may be relevant to the predominant mucosal transmission of R5 virus and the rapid depletion of GALT T lymphocytes at the early stages of HIV-1 infection.

Although R5 replicated more efficiently in rectosigmoid compared with tonsillar tissue, in both tissues X4 targets, the CXCR4-positive CD4 T lymphocytes, comprise the majority of the CD4 T cell population [21,33,34], and both tissues support X4 infection. With the improvement in staining techniques, it becomes apparent that virtually all the CD4 T cells are potential targets for X4.

In addition to the chemokine receptors, chemokines themselves may modulate HIV-1 infection as one of the first lines of antimicrobial defence. The upregulation of chemokines occurs in human tonsillar tissue *ex vivo* upon X4 but not R5 HIV-1 infection. It thus appears that at a local level, secondary lymphoid tissue is capable of mounting a defence, although not broadly effective, as X4 largely upregulates MIP-1α, MIP-1β and RANTES that do inhibit R5 rather than X4 [35].

In rectosigmoid tissue, the basal level of chemokines is greater than in tonsillar tissue, and might be thought to be an obstacle to R5 infection. Nevertheless, as shown here, rectosigmoid tissue is readily infected by R5. It is conceivable that, because of the high expression of CCR5, the basal levels of CCR5 ligands in rectosigmoid tissue might not saturate this receptor to inhibit HIV-1 entry. In addition, X4 infection in rectosigmoid tissue does not upregulate chemokine secretion (as it does in the tonsils). This lack of upregulation is not because rectosigmoid tissue is unable to upregulate chemokines further as we demonstrated with phytohemaglutinin stimulation. The inability of rectosigmoid tissue to upregulate cytokines in response to HIV-1 infection may render it more vulnerable to HIV-1 than secondary lymphoid tissue.

Also, it is conceivable that the spikes of X4 in secondary lymphoid tissue demonstrated by Mullins *et al*. [36] may upregulate CC chemokines, thus suppressing R5, whereas in gut tissue, where X4 fails to upregulate chemokine release, there is no such suppressive mechanism.

In conclusion, by comparing tonsillar and rectosigmoid tissue susceptibility to R5 and X4 HIV-1, we showed that the latter is more susceptible to R5 infection than the former. This difference seems to be related to the much greater expression of CCR5 co-receptors in rectosigmoid than in tonsillar tissue. The majority of rectosigmoid CD4 lymphocytes still express CXCR4, however, either alone or in combination with CCR5, and therefore this tissue is also readily infected with X4 *ex vivo*. This strongly suggests that co-receptor expression alone is not sufficient to explain the R5 predominance seen in early infection. Undoubtedly, there are additional mechanisms that serve as partial 'gatekeepers' restricting X4 infection *in vivo* [27].

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ANEXO II

OUTROS ARTIGOS PUBLICADOS DURANTE O DOUTORADO - II

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HIV-1 induced activation of CD4+ T cells creates new targets for HIV-1 infection in human lymphoid tissue ex vivo

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HIV-1–induced activation of CD4$^+$ T cells creates new targets for HIV-1 infection in human lymphoid tissue ex vivo

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We demonstrate mechanisms by which HIV-1 appears to facilitate its own infection in ex vivo–infected human lymphoid tissue. In this system, HIV-1 readily infects various CD4$^+$ T cells, but productive viral infection was supported predominantly by activated T cells expressing either CD25 or HLA-DR or both (CD25/HLA-DR) but not other activation markers: There was a strong positive correlation ($r = 0.64$, $P = .001$) between virus production and the number of CD25$^+$/HLA-DR$^+$ T cells. HIV-1 infection of lymphoid tissue was associated with activation of both HIV-1–infected and uninfected (bystanders) T cells. In these tissues, apoptosis was selectively increased in T cells expressing CD25/HLA-DR and p24gag but not in cells expressing either of these markers alone. In the course of HIV-1 infection, there was a significant increase in the number of activated (CD25$^+$/HLA-DR$^+$) T cells both infected and uninfected (bystander). By inducing T cells to express particular markers of activation that create new targets for infection, HIV-1 generates in ex vivo lymphoid tissues a vicious destructive circle of activation and infection. In vivo, such self-perpetuating cycle could contribute to HIV-1 disease. (Blood. 2008;111: 699-704)

Introduction

CD4$^+$ T lymphocytes are the major target for HIV-1 infection,[1] and their loss is the hallmark of HIV-1 disease.[2-4] It is well established that the critical event of HIV-1 infection occurs in lymphoid tissue where T lymphocytes expressing CD4 constitute a highly heterogeneous population different in many parameters, in particular, their activation status.[5-8] Unlike single cell cultures in vitro, the tissue microenvironment provides conditions for both activated and nonactivated cells to be productively infected.[9,10] Nevertheless, CD4$^+$ T-cell activation is thought to be a major factor in facilitating HIV-1 infection of these cells.[11,12] This, and several other observations, have led to the widely accepted hypothesis that tissue activation is a major force, driving HIV-1 disease progression (for review, see Grossman et al[13,14]). The fraction of activated T lymphocytes and other cell types is increased in HIV-1–infected patients, and we have recently documented distorted activation pattern of lymphocytes in lymph nodes and tonsils from HIV-1–infected patients.[15] However, the patterns of activation determining cell susceptibility to productive HIV-1 infection, the contribution of nonactivated cells to the viral load, and the relationship between activation status and cell loss in lymphoid tissues remain largely unknown, in part because of the lack of an adequate experimental model to address these problems.

Here, to reveal mechanisms connecting cell activation and HIV-1 infection, we used ex vivo–infected human lymphoid tissues. These tissues support productive HIV-1 infection ex vivo without exogenous activation[16,17] that is needed to efficiently infect peripheral blood mononuclear cells (PBMCs); such conditions are unlikely to reflect the conditions of cell activation in vivo even remotely. Moreover, in ex vivo tissues, similar to in vivo,[10,18] both activated and nonactivated cells become productively infected,[19] providing an experimental system to address some aspects of cell activation in HIV-1–infected human lymphoid tissue.

By comparing matched infected and noninfected lymphoid tissues from individual donors, we demonstrated here that viral load in this system depends on the number of activated target cells, but only of a particular pattern, CD25$^+$/HLA-DR$^+$. Furthermore, we found that viral infection mobilizes new HIV-1 cell targets by activating uninfected cells to express the very same pattern of markers that is associated with the efficient HIV-1 replication. HIV-1 infection of these cells drives them into apoptosis. Thus, HIV-1 creates in lymphoid tissue ex vivo a cycle of activation and infection that is also directly associated with tissue destruction. A similar pathogenic cycle operating in vivo might partly explain the role of cell activation in disease progression.

Methods

The use of anonymous surgical waste was approved by the National Institute of Child Health and Human Development and Children's National Medical Center Institutional Review Boards.

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Lymphoid tissue histocultures and HIV-1 infection

Tonsillar tissue obtained at the Children's National Medical Center (Washington, DC) during routine therapeutic tonsillectomy according to an IRB-approved protocol were dissected into approximately 3-mm blocks and placed on top of collagen sponge gels. Tissue blocks were infected with the HIV-1 X4 variants LAI.04 ($X4_{LAI.04}$) and NL4.3 ($X4_{NL4.3}$) or with the R5 variants SF162 ($R5_{SF162}$) and AD8 ($R5_{AD8}$; National Institutes of Health AIDS Research Program), as described earlier.[16,17] In a typical experiment, 3 to 5 μL of clarified virus-containing medium (approximately 300 $TCID_{50}$ per block) were applied to the top of each tissue block. In each experiment, we compared with matched tissues, ie, tissue blocks obtained from the same donor. Tissue blocks (both HIV-1–infected and matched uninfected) were cultured for 12 days in RPMI 1640 (GibcoBRL; Invitrogen, Carlsbad, CA) containing 15% heat-inactivated fetal calf serum (Summit Biotechnology, Fort Collins, CO), nonessential amino acids (1 mM), sodium pyruvate (1 mM), L-glutamine (292 μg/mL), amphotericin B (2.5 μg/mL; GibcoBRL; Invitrogen), and gentamicin (50 μg/mL; Quality Biological, Rockville, MD). HIV-1 replication was assessed as described in "Evaluation of HIV-1 replication."

Evaluation of HIV-1 replication

We assessed productive infection by measuring HIV-1 antigen p24gag accumulated in the culture medium during the 3 days between the successive medium changes, using p24gag antigen enzyme-linked immunosorbent assay (ELISA) detection kits (Perkin Elmer, Wellesley, MA; and Beckman Coulter, Miami, FL).

Flow cytometry

Single-cell suspensions were prepared from tissue blocks by digestion with Collagenase IV (GibcoBRL) at 5 mg/mL in RPMI 5% fetal calf serum for 30 minutes, followed by a wash in staining buffer (phosphate-buffered saline supplemented with 2% normal mouse serum, Gemini Bioproducts, West Sacramento, CA). Lymphocytes were identified according to their light-scattering properties and then analyzed for the expression of activation markers. To determine the proportion of infected cells, we washed the cells
3 times and stained them with different combinations of the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD25, anti-CD69, anti-CD38, anti-HLA-DR, and anti-CD95 coupled to a combination of fluorochroms: fluorescein isothiocyanate (FITC), phycoerythrin (PE)–Alexa610, PE-Cy7, PE-Cy5, PerCP-Cy5.5, or APC (Caltag Laboratories, Burlingame, CA; Becton Dickinson, San Jose, CA). After surface staining, the cells were permeabilized with Fix&Perm reagent (Caltag) and stained with anti-HIV-1-p24gag monoclonal antibody (KC57-RD1(PE), Beckman Coulter) and with APO 2.7 (PE-Cy5; Beckman Coulter) a monoclonal antibody specific for an early apoptosis mitochondrial antigen. Cells washed and fixed in phosphate-buffered saline, containing 4% formaldehyde, were acquired on an LSRII flow cytometer equipped with 355, 488, 532, 407, and 638 nm LASER lines using DIVA 4.1.2 software. Data were analyzed with FlowJo version 8.3 software (Tree Star, Ashland, OR). The percentage of infected T cells is reported for events gated on $CD3^+$ cells with anti-HIV-1-p24gag-PE for intracellular staining.

Statistical analysis

Data obtained with tissue (27 or 54 tissue blocks for each experimental condition) from one donor were considered as one experiment (n). Because both the levels of viral replication and the proportions of cells in various leukocyte subsets varied from donor to donor,[17,20] the results of different experiments were normalized per number of blocks (either uninfected or infected), averaged, and analyzed statistically. Statistical analysis performed on the normalized results data included the calculation of mean, SEM, and *P* values by use of a multiple comparison test (2-way analysis of variance test). The significance level was set as *P* at 0.5 or less, and the actual *P* values are indicated for each series of experiments. Statistical analysis of p24gag ELISA data was performed with Deltasoft version 3.0 software (BioMetallics, Princeton, NJ), by combining data from 3 dilutions and calculating a weighted interpolated p24gag concentration and SEM using a 4-parameter fitting algorithm.

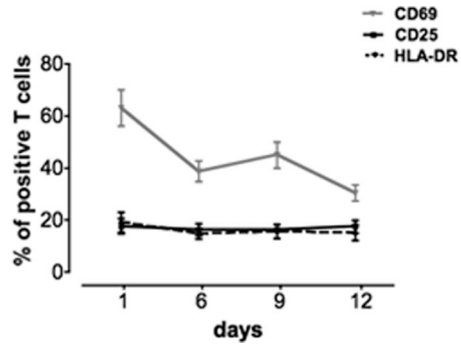

Figure 1. Kinetics of expression of different activation markers in human lymphoid tissue ex vivo. Tissues from 8 to 22 different donors were immunostained for different activation markers (CD69, CD25, and HLA-DR). The graphs represent the means (± SEM) of T cells expressing these markers at days 1, 6, 9, and 12 after the beginning of culture. Shown are the fractions of cells positive for CD25, CD69, and HLA-DR expression.

Results

Activated T cells in human lymphoid tissue ex vivo

We evaluated the number of activated T cells in cultured noninfected blocks of human tonsils. In this study, we followed activation by expression of CD69 (early activation marker[21,22]) and of CD25 and HLA-DR (late activation markers[23,24]; Figure 1). Flow cytometric analysis on day 1 in culture revealed that, on average, 63 plus or minus 6.9% of T cells expressed CD69 (n = 13). This fraction is not different from that observed in uncultured lymphoid tissue after surgery (data not shown). The other activation markers were expressed to a lower extent: HLA-DR was expressed on 19 plus or minus 3.8% (n = 12) and CD25 on 17.5 plus or minus 3% (n = 12) of T cells (Figure 1).

With time, the number of cells expressing CD69 dramatically decreased: on day 6 in culture these cells constituted 38.7 plus or minus 3.9% (n = 22) of the T cells and on day 12 this number dropped to 30 plus or minus 3% (n = 22; $P = .002$). In contrast, the numbers of T cells that expressed CD25 and HLA-DR remained at approximately the same levels as on day 1, and on day 12 these cells constituted 15 plus or minus 3% (n = 22) and 17 plus or minus 2.5% (n = 22) of the total number of T cells, respectively. In addition, expression of other activation markers (CD38 and CD95) was also stable over the 12 days of culture (not shown). CD38 was expressed in 26.1 plus or minus 4.7% (n = 5) of T cells at day 1 and 16 plus or minus 4.9% (n = 5) at day 12 ($P = .15$). CD95 was expressed in 28.6 plus or minus 4% of T cells at day 1 and in 28 plus or minus 7% at day 12 ($P = .1$). Thus, in uninfected human lymphoid tissue ex vivo, the pattern of T-cell activation changes in time with the decrease in CD69 expression, whereas the levels of expression of CD25, CD38, CD95, and HLA-DR remain stable.

HIV-1 infection in activated and nonactivated tissue $CD4^+$ T cells

Next, we investigated the relative contributions of differently activated $CD4^+$ T cells to viral production. We stained cells for activation markers (CD25/HLA-DR) and for the intracellular viral antigen p24gag. Flow cytometric analysis of these cells revealed that both $CD25^+$/$HLA\text{-}DR^+$ $CD4^+$ T cells and $CD25^-$/$HLA\text{-}DR^-$ $CD4^+$ T cells were productively infected (Figure 2).

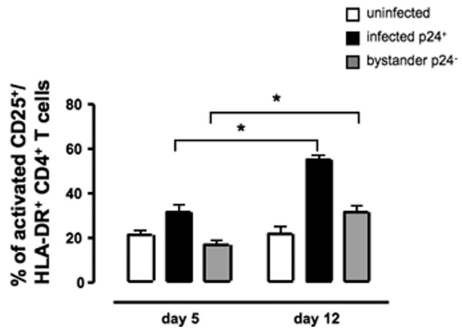

Figure 2. Activation in infected and uninfected CD4⁺ T cells in HIV-1–infected tissues. Tissue blocks from 3 different donors (54 blocks for each data point) were infected with $X4_{LAI.04}$. $CD4^+$ T cells from infected and matched uninfected tissues were stained for activation markers (HLA-DR and CD25) and for p24gag and analyzed by flow cytometry. The graphs represent the averages (± SEM) of $CD25^+/HLA\text{-}DR^+$ $CD4^+$ T cells at days 5 and 12 after infection. $CD25^+/HLA\text{-}DR^+$ $CD4^+$ T cells in uninfected control tissue. $CD25^+/HLA\text{-}DR^+$ $CD4^+$ T cells were divided in $X4_{LAI.04}$ infected tissue in productively infected cells ($p24gag^+$) and bystander cells ($p24gag^-$). * Represents significant differences for a nonparametric paired T test.

We investigated whether activated and nonactivated cells are infected with differential efficiency. The ratios of infected ($p24gag^+$) to uninfected ($p24gag^-$) $CD4^+$ T cells among activated ($CD25^+/HLA\text{-}DR^+$) and nonactivated ($CD25^-/HLA\text{-}DR^-$) subsets were 1.9 plus or minus 0.18 and 0.97 plus or minus 0.16, respectively ($n = 5$, $P = .005$). Thus, in ex vivo–infected human lymphoid tissue, an activated $CD4^+$ T cell is twice as likely as a nonactivated $CD4^+$ T cell to be productively infected with HIV-1.

Tissue T-cell activation and HIV-1 production

Here, we investigated whether there is a correlation between the efficiency of viral production in tissue as evaluated from p24gag accumulation ("viral load") and the number of activated T cells (Figure 3). We evaluated the proportion of T cells expressing either CD69 or CD25/HLA-DR at day 6 when the infection is readily detectable in the culture medium bathing blocks of tissues. In matched tissues infected with either $X4_{LAI.04}$ (Figure 3A,C) or $R5_{SF162}$ (Figure 3B,D), we evaluated the cumulative production of p24gag over the duration of the experiment.

There was a strong positive linear correlation between both $X4_{LAI.04}$ and $R5_{SF162}$ viral loads and the fraction of T cells that expressed CD25/HLA-DR ($r = 0.64$, $P < .001$, $n = 34$, for $X4_{LAI.04}$, and $r = 0.5$, $P = .01$, $n = 22$, for $R5_{SF162}$; Figure 3A,B). In contrast, there was no correlation between these viral loads and the fraction of T cells expressing either CD38, CD95 (data not shown) or CD69 at day 6 ($r = -0.2$ for $X4_{LAI.04}$ and $r = -0.1$ for $R5_{SF162}$; $n = 10$; $P = .57$ and $P = .71$, respectively). Because of the fast down-regulation of CD69 in culture (Figure 1), we correlated the expression of CD69 in T cells at day 1 with viral loads and found a negative correlation for $X4_{LAI.04}$ ($r = -0.75$, $P = .01$, $n = 10$; Figure 3C) and no correlation with R5 HIV-1-viral load ($r = -0.28$, $P = .32$, $n = 13$; Figure 3D).

We performed the same experiments with 2 other viral strains, $X4_{NL4\text{-}3}$ and$R5_{AD8}$. Akin to the correlation between the fraction of activated T cells and $X4_{LAI.04}$ replication, we observed a negative trend between $X4_{NL4\text{-}3}$ replication and the fraction of T cells that expressed CD69 at day 1 ($r = -0.54$, $n = 4$, $P = .45$), whereas there was no correlation between $R5_{AD8}$ replication and expression of this marker ($r = -0.1$, $n = 4$, $P = .89$). There were positive trends between replication of these viruses and the fractions of T cells that expressed CD25/HLA-DR ($r = 0.59$ for $X4_{NL4\text{-}3}$ and $r = 0.63$ for $R5_{AD8}$; $n = 6$; $P = .21$ and $P = .12$, respectively).

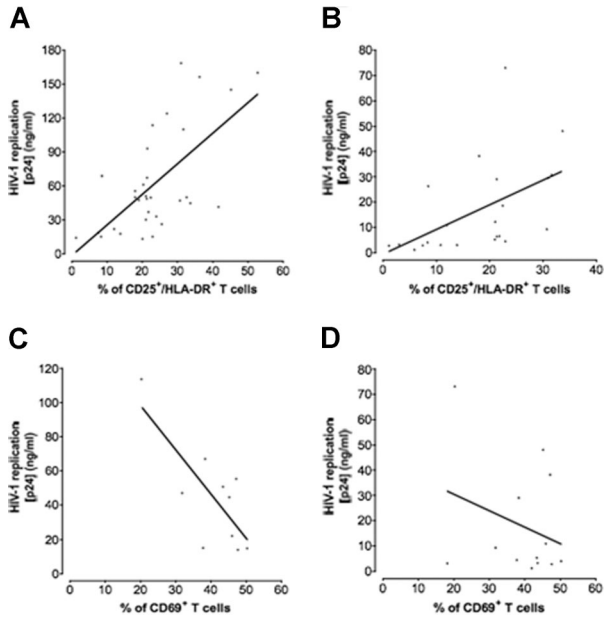

Figure 3. Correlation between p24gag production and T-cell activation. Tissues from 8 to 32 different donors were infected with $X4_{LAI.04}$ or $R5_{SF162}$, and for each data point the amount of p24gag accumulated in culture medium bathing 54 infected tissue blocks was measured by p24gag ELISA. Matched uninfected tissue blocks were used as controls. T cells of either $CD69^+$ or $CD25^+/HLA\text{-}DR^+$ phenotype were considered as activated. The graphs represent the linear regression between the fraction of activated T cells in noninfected tissues and the maximal p24gag production in the matched infected tissue. (A) $X4_{LAI.04}$ production correlated with $CD25^+/HLA\text{-}DR^+$ T cells. (B) $R5_{SF162}$ production correlated with $CD25^+/HLA\text{-}DR^+$ T cells. (C) $X4_{LAI.04}$ production correlated with $CD69^+$ T cells. (D) $R5_{SF162}$ production correlated with $CD69^+$ T cells.

To investigate whether there is a correlation between the viral load and the number of activated $CD4^+$ T cells, we evaluated the proportion of $CD4^+$ T cells expressing CD25/HLA-DR. As in the case of the correlation described above for activated T cells taken together, there was a positive linear correlation between the $X4_{LAI.04}$ viral load and the fraction of CD4 T cells that expressed CD25/HLA-DR ($r = 0.70$, $n = 7$, $P = .004$). Thus, in human lymphoid tissue ex vivo, the number of $CD4^+$ T cells expressing CD25/HLA-DR but not CD69 is a major positive correlate of the efficiency of HIV-1 replication.

HIV-1–driven activation of CD4⁺ T cells

We studied whether infection of lymphoid tissue by HIV-1 modulates their activation status. On day 5 after infection, on average 90 plus or minus 2% of $CD4^+$ T cells were uninfected ($n = 5$). As shown in Figure 2, on day 5 after infection, the presence of infected $CD4^+$ T cells did not significantly change the fraction of $CD25^+/HLA\text{-}DR^+$ cells among uninfected $CD4^+$ T cells residing in the same tissue (bystander cells): they constituted 20.8 plus or minus 1.9% and 16.6 plus or minus 1.8% in uninfected and infected tissues, respectively ($n = 5$, $P = .14$). Thus, on day 5 after infection, the frequency of activated bystander $CD4^+$ T cells remained similar to that in matched uninfected control tissues ($P = .14$).

In contrast, on day 12 after infection, the fractions of activated cells among bystander and among productively infected $CD4^+$ T cells increased to 31.3 plus or minus 2.6% ($n = 5$, $P = .001$) and 54.6 plus or minus 2% ($n = 5$, $P < .001$), respectively (Figure 2). In uninfected control tissue, however, the fraction of activated cells did not change and remained 21.5 plus or minus 2.9% ($n = 5$, $P = .85$) at day 12.

Table 1. Preferential depletion of activated CD4⁺ T cells

Activation status by day	Frequencies of apoptotic cells, %	
	Uninfected	Infected
Activated		
Day 5	8 ± 2	13 ± 0.5
Day 12	10.5 ± 2	31 ± 3
*P**, day 12	.3	.003
Nonactivated		
Day 5	6 ± 1	6 ± 0.6
Day 12	5 ± 2	10 ± 4
*P**, day 12	.054	.27

* *P* values are calculated for a paired *t* test measured between day 5 and day 12.

We also calculated the ratio of activated to nonactivated CD4⁺ T cells for the 3 populations: p24gag⁻ in uninfected, p24gag⁻ and p24gag⁺ in infected tissues. We found that there was no change in uninfected tissue. This ratio (activated to nonactivated CD4⁺ T cells) was 1 plus or minus .01 (n = 5) in uninfected tissue, whereas for infected tissue, these ratios were on average 1.9 plus or minus 0.2 for p24gag⁻ CD4⁺ T cells (n = 5, $P < .001$) and 1.8 plus or minus 0.2 for p24gag⁺ CD4⁺ T cells (n = 5, $P = .002$). Thus, on day 12 there was a higher proportion of both bystander and HIV-1 productively infected activated T CD4⁺ cells suggesting that HIV-1 drives activation of CD4⁺ T cells.

Preferential apoptosis of activated CD4⁺ T cells

As in the case of infection in vivo, productive HIV-1 infection in ex vivo tissue results in the depletion of CD4⁺ T cells. Here, we studied the relation between the activation status of an infected cell and its fate. We evaluated the fractions of CD4⁺ T cells expressing an early apoptotic marker: the mitochondrial antigen Apo2.7, and the activation markers CD25⁺/HLA-DR⁺ in HIV-1–infected and in matched control tissues (Table 1). In uninfected tissues, the frequencies of apoptosis in CD4⁺ T cells of the CD25⁺/HLA-DR⁺ subset were not significantly different from that in CD4⁺ T cells of the CD25⁻/HLA-DR⁻ subset (8 ± 2% vs 6 ± 1% and 10.5 ± 2% vs 5 ± 2% for days 5 and 12, respectively; n = 3; $P = .3$ and $P = .054$). In infected tissues on day 5 after infection, the Apo2.7⁺ cells constituted 13 plus or minus 0.5% of the CD25⁺/HLA-DR⁺ CD4⁺ T cells, and this fraction increased significantly on day 12, on average to 31 plus or minus 3% (n = 3, $P = .003$). In contrast, apoptosis was not significantly increased among CD25⁻/HLA-DR⁻ CD4⁺ infected T cells: on day 5, Apo2.7⁺ cells constituted 6 plus or minus 0.6%, and on day 12, 10 plus or minus 4%, of these cells (n = 3, $P = .27$). Thus, infection with HIV-1 selectively increased apoptosis among activated CD4⁺ T cells.

Discussion

In this work, we investigated in an ex vivo model of human lymphoid tissue the role of cell activation in HIV-1 replication because this activation was hypothesized to be the driving force of HIV-1 disease in general[13,14,25-30] and T-cell turnover in particular.[31-33] The system of ex vivo–infected human lymphoid tissue used in our experiments is more adequate to address this question than conventional cell culture systems in which blasting PBMCs with phytohemagglutinin/interleukin-2 changes their activation status. As reported earlier and confirmed here, ex vivo HIV-1 inoculation of tissue blocks results in efficient productive infection[34-37] without exogenous activation or stimulation, and therefore both activation and infection occur in our system under conditions that resemble in many ways those prevailing in lymphoid tissue in vivo. However, ex vivo HIV-1–infected tissues do not reflect some important aspects of HIV-1 pathogenesis, eg, changes in lymphocyte circulation, aberrant lymphocyte proliferation, and various systemic factors. These limitations should be kept in mind when extrapolating our results to an in vivo situation. The kinetics of HIV-1 replication in ex vivo lymphoid tissue evaluated by the release of p24gag into the medium is highly reproducible.[34,38] However, the absolute level of viral infection significantly varied from donor to donor (see also Glushakova et al,[34] Penn et al,[38] and Grivel et al[39]). Here we found that the efficiency of viral infection is determined by the tissue T-cell expression of the particular activation markers. For this study, we monitored expression of CD69, CD25/HLA-DR, CD38, and CD95. In the course of a 12-day experiment, the expression of CD25 and HLA-DR, as well as of CD95 and CD38 was stable, whereas that of CD69 was downregulated. The rapid decrease of CD69 expression may reflect the physiology of T-cell circulation in vivo. Indeed, in secondary lymphoid tissues (ie, tonsils, lymph nodes), T cells express CD69 for 18 to 24 hours[40] while they are retained in lymph nodes.[41,42] Later, T cells lose CD69 and move out of the lymphoid tissue. In isolated tissue blocks in which the normal cell trafficking is disrupted, these cells nevertheless down-regulate CD69 at the same time they would do it in vivo.

We studied whether the expression of these markers that reflect various aspects of tissue activation status determines the viral load on tissue HIV-1 infection. Our analysis revealed in this system a strong linear correlation between HIV-1 production (both X4 and R5 variants) and the number of CD25⁺/HLA-DR⁺ T cells at the time of viral spread (day 6 after infection).[34] In contrast, there was no correlation between HIV-1 production and expression of either CD69 or CD38, or CD95 at that time. However, because CD69 expression is transient and is lost within 72 hours of its initial appearance, CD69 measurement after 6 days of culture ex vivo does not reflect the activation status of the tissue at the time of HIV-1 infection (day 1). Therefore, we measured the expression of CD69 at the day of infection and found no correlation (for R5 variants) and a negative correlation (in case of X4 variants) with HIV-1 production.

It seems that in human lymphoid tissue, expression of the early activation marker CD69 is the attribute of tissue less susceptible to productive HIV-1 infection, whereas expression of late activation markers (CD25/HLA-DR) is the attribute of tissue more susceptible to productive HIV-1 infection. Unfortunately, the physiologic role of most of the activation markers is not known. Likewise, the expression of a particular marker CD69 or CD25/HLA-DR can be either a cause or consequence of the susceptibility to HIV-1 replication, whose underlying molecular mechanisms need to be addressed in future studies. Nevertheless, our results clearly establish that in lymphoid tissue a particular pattern, rather than general activation, is required for efficient HIV-1 replication. This implies that the use of the term "activation" in the context of HIV-1 infection requires qualification of which markers have been used to define activation. The role of the molecules that are now used as activation markers for most cases remains unclear. Future studies may reveal that the molecular mechanisms that lead to expression of particular set of markers are different and thus would define the diverse processes that are now collectively and vaguely referred to as "activation."

Unlike in various in vitro systems based on isolated cell cultures, even nonactivated T cells in human lymphoid tissue

(both in vivo and ex vivo) support productive HIV-1 infection.[19,35,37,43] However, we found here that the frequency of infection, as evaluated from intracellular p24gag staining, was twice higher among activated HLA-DR$^+$/CD25$^+$ T cells than among nonactivated HLA-DR$^-$/CD25$^-$ ones. Moreover, in contrast to the correlation of the number of activated T cells with viral production, there was no such correlation between HIV-1 replication and the number of nonactivated T cells. In the future, it would be interesting to directly compare the individual productivity of an activated and a nonactivated infected cell. Nevertheless, our data indicate that infected, nonactivated T cells produce much less virus than activated cells, as was previously reported in mucosal tissue and in PBMCs.[9,18]

Remarkably, HIV-1 infection seems to facilitate the very same pattern of lymphoid tissue activation that is associated with enhanced HIV-1 replication. The number of T cells of this phenotype increased with the duration of tissue infection not only among HIV-1–infected cells but also among uninfected bystander cells. A possible mechanism of T-cell activation in HIV-1–infected cells would occur via production of the Nef and Tat proteins, which activate both NF-AT and NF-κB, was reported earlier,[18,44] whereas the mechanism of activation of bystander cells remains to be fully understood. Whichever this mechanism, such HIV-1–induced activation of bystander cells should promote viral spreading by creating a pool of new viral targets that on infection produce virus at a higher rate. Both in in vivo and in ex vivo tissues, HIV-1 infection of T cells leads to their death through apoptosis.[45-50] The resultant kinetics of viral replication depends on how quickly apoptosis occurs in infected and activated cells, at which stage of apoptosis HIV-1 production is shut down, and how HIV-1 propagates through the tissue in the course of experiment.

Do activated and nonactivated T cells survive differentially in productively infected tissues? In the present work, we monitored the coexpression of an early apoptotic marker, the mitochondrial antigen Apo2.7,[51,52] and the activation markers HLA-DR and CD25. Our results show that productively infected activated cells tend to enter apoptosis, whereas productively infected but nonactivated T cells do not express this apoptotic marker. Thus, infection of T cells does not seem to be sufficient to efficiently draw them into apoptosis. This may be simply related to the low amount of the virus produced by nonactivated cells but may also depend on yet unknown processes that are triggered by HIV-1 exclusively in CD25$^+$/HLA-DR$^+$ CD4 T cells. Neither does activation alone seem to be sufficient to induce apoptosis because, in our experiments in uninfected tissues, apoptotic frequencies among activated T cells were not different from that among nonactivated T cells. It seems that a combination of productive infection and activation is necessary for efficient T-cell apoptosis. Therefore, the frequency of activated infected T cells in tissues reported in our results may be underestimated because of their death before the time point of our analysis. If so, their contribution to HIV-1 production and T-cell depletion is even more significant.

Extrapolation of this observation to the in vivo situation suggests that nonactivated T cells are producing HIV-1 with low efficiency but survive longer than activated infected cells and therefore may contribute to the long-lived viral reservoir. In general, immunoactivation may be one of the critical factors for T-cell depletion in HIV-1–infected individuals.[13,53]

In summary, the amount of T cells of a particular activation pattern determines the efficiency of viral production in human lymphoid tissue. HIV-1 infection induces tissue cells to express this particular pattern of activation markers, both on infected and on the uninfected bystander T cells, thus creating new HIV-1 cell targets. Although activated and nonactivated cells support productive HIV-1 infection, the size of the latter pool is smaller than that of the former and the number of activated T cells productively infected with HIV-1 is the major correlate for viral load. A combination of HIV-1 infection and activation, but neither of these factors alone, is sufficient to drive cells into apoptosis.

In conclusion, our results are in general agreement with the in vivo observations on the central role of cell activation in HIV-1 disease but go beyond confirmation of this phenomenon in ex vivo tissues by demonstrating the existence of an activation–infection cycle that locally enhances the replication of HIV-1 in lymphoid tissues and drives cells into apoptosis. Such a cycle operating in vivo would facilitate viral pathogenesis in infected tissues leading to AIDS. The ex vivo tissue system can be used to further explore the details of that cycle; targeting individual elements of this cycle may become part of an anti-HIV-1 strategy.

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Authorship

Contribution: A.B., I.H., L.B.M., and J.-C.G. wrote the paper and designed the research; and A.B., S.J.H., C.V., C.E.C., A.L., and E.R. performed research.

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