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# Caracterização de precursores de linfócitos T CD8$^{+}$ residentes de memória no tecido genital durante a fase aguda da infecção por HSV-2 e impacto da terapia com aciclovir

UFCSPA
Universidade Federal de Ciências da Saúde de Porto Alegre

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Dissertação submetida ao Programa de Pós-Graduação em Patologia da Universidade Federal de Ciências da Saúde de Porto Alegre como requisito para a obtenção do grau de Mestre.

Orientador: Dr. Luiz Carlos Rodrigues Junior
Coorientador: Dr. Marvin Paulo Lins

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## RESUMO

**Introdução:** Linfócitos T $CD8^+$ desempenham papel central no controle da infecção pelo HSV-2. Entre eles, as células T residentes de memória (TRM) contribuem para a imunovigilância local e para o controle de reativações virais. A diferenciação dessas células depende da disponibilidade de antígeno no tecido. O aciclovir, principal antiviral utilizado contra o HSV-2, reduz a replicação viral e pode influenciar a ativação e diferenciação de linfócitos T durante a fase aguda da infecção. **Objetivo:** Avaliar como o controle da carga viral pelo aciclovir influencia a geração de precursores de células T $CD8^+$ TRM na fase aguda da infecção por HSV-2. **Metodologia:** Camundongos fêmeas C57BL/6 foram infectados por via intravaginal com diferentes doses de HSV-2 ($7,5\times10^3$, $1,5\times10^4$ e $3\times10^4$ PFU/ml). Em uma segunda etapa, animais infectados receberam tratamento com aciclovir. A evolução clínica foi acompanhada por avaliação de peso, lesão genital e sobrevivência. No 14º dia pós-infecção, tecido genital, linfonodos drenantes e baço foram coletados para análise por citometria de fluxo, incluindo células T $CD8^+$ e os marcadores CD44, PD1, CD69, CD103 e CD49a. **Resultados:** A infecção induziu resposta imune local dependente da carga viral, com aumento de células T $CD8^+$ ativadas e de marcadores associados à retenção tecidual. O tratamento com aciclovir reduziu a morbidade, com menor perda de peso, menor escore de lesão e maior sobrevivência. Além disso, reduziu a frequência de células T $CD8^+$ ativadas, a expressão de PD-1 e a frequência de células $CD69^+$, $CD103^+$ e $CD49a^+$ no tecido genital. Não foram observadas alterações relevantes no baço e nos linfonodos drenantes. **Conclusão:** A redução precoce da carga viral pelo aciclovir modula a ativação de células T $CD8^+$ e a geração de precursores de TRM durante a fase aguda da infecção por HSV-2. Esses resultados

reforçam a importância da disponibilidade de antígeno na regulação da resposta imune local.

**Palavras-chave:** HSV-2; células T residentes de memória; linfócitos T $CD8^+$; aciclovir; carga viral; mucosa genital.

**Objetivos de desenvolvimento sustentável:** ODS3 - Saúde e Bem-estar.

## ABSTRACT

**Introduction:** $CD8^+$ T lymphocytes play a vital role in controlling HSV-2 infection. Among them, tissue-resident memory T cells (TRM) contribute to local immune surveillance and the control of viral reactivations. The differentiation of these cells depends on antigen availability within the tissue. Acyclovir, the main antiviral drug used against HSV-2, reduces viral replication and may influence T cell activation and differentiation during the acute phase of infection. **Objective:** To evaluate how acyclovir-mediated viral load control influences the generation of $CD8^+$ TRM precursor cells during the acute phase of HSV-2 infection. **Methods:** Female C57BL/6 mice were infected intravaginally with different doses of HSV-2 ($7{,}5\times10^3$, $1{,}5\times10^4$, and $3\times10^4$ PFU/ml). In a second experimental phase, infected animals received therapeutic acyclovir treatment. Clinical progression was monitored through body weight, genital lesion score, and survival. Fourteen days post-infection, genital tissue, draining lymph nodes, and spleens were collected for flow cytometric analysis, including the evaluation of CD8+ T cells and the expression of CD44, PD1, CD69, CD103, and CD49a. **Results:** Infection induced a viral load-dependent local immune response, characterized by increased frequencies of activated $CD8^+$ T cells and cells expressing markers associated with tissue retention. Acyclovir treatment reduced disease severity, as evidenced by decreased weight loss, lower lesion scores, and increased survival. In addition, treatment reduced the frequency of activated $CD8^+$ T cells, PD-1 expression, and the frequency of $CD69^+$, $CD103^+$, and $CD49a^+$ cells in the genital tissue. No relevant alterations were observed in the spleen or draining lymph nodes. **Conclusion:** Early reduction of viral load by acyclovir modulates $CD8^+$ T cell activation and the generation of TRM precursors during the acute phase of HSV-2 infection.

These findings highlight the importance of antigen availability in regulating the local immune response.

**Keywords:** HSV-2; tissue-resident memory T cells; $CD8^+$ T cells; acyclovir; viral load; genital mucosa.

**Sustainable Development Goals:** SDG 3 – Good Health and Well-being.

## ORGANIZAÇÃO DA DISSERTAÇÃO

Este trabalho de mestrado foi desenvolvido no Laboratório de Imunovirologia da UFCSPA, em colaboração com o grupo de Imunoterapia da mesma instituição. Inicialmente, o projeto tinha como objetivo realizar uma caracterização abrangente da resposta de linfócitos T CD8 residentes em tecidos (CD8 TRMs) durante a infecção por HSV-2, contemplando tanto a fase aguda quanto a fase de memória imunológica.

Entretanto, por se tratar de uma linha de pesquisa recente em nosso grupo e ainda pouco explorada no Brasil, foi necessário dedicar um período significativo à padronização e validação dos protocolos experimentais. A caracterização fenotípica e funcional de populações de células T residentes em tecidos envolve procedimentos metodológicos complexos, especialmente considerando o tempo disponível para a execução de um projeto de mestrado.

Dessa forma, ao longo deste trabalho foram implementadas e validadas as principais técnicas necessárias para a análise dessas populações celulares. Em razão disso, a presente dissertação concentra-se na caracterização da resposta durante a fase aguda da infecção. Os resultados obtidos permitiram a elaboração de um pré-manuscrito (artigo), que será posteriormente complementado com estudos da fase de memória no âmbito do projeto de doutorado de um novo integrante do grupo de pesquisa.

Adicionalmente, foi produzido um artigo de revisão sobre o papel dos linfócitos T CD8 residentes em tecidos nas infecções virais, do qual o mestrando é coautor. Essa publicação atende ao requisito institucional de produção científica para a obtenção do título de Mestre.

## LISTA DE ABREVIATURAS

APC – Antigen-Presenting Cell

BHLHE40 – Basic Helix-Loop-Helix Family Member E40

BLIMP-1 – B Lymphocyte-Induced Maturation Protein 1

CCR7 – C-C Chemokine Receptor Type 7

CD – Cluster of Differentiation

CD62L – Cluster of Differentiation 62 Ligand

cGAS – Cyclic GMP-AMP Synthase

CpG – Cytosine-Phosphate-Guanine

CXCL9 – C-X-C Motif Chemokine Ligand 9

CXCL10 – C-X-C Motif Chemokine Ligand 10

CXCL16 – C-X-C Motif Chemokine Ligand 16

CXCR3 – C-X-C Chemokine Receptor Type 3

CXCR6 – C-X-C Chemokine Receptor Type 6

DC – Dendritic Cell

DNA - Deoxyribonucleic Acid

EOMES – Eomesodermin

HOBIT – Homolog of Blimp-1 in T Cells

HSV – Herpes Simplex Virus

HSV-1 – Herpes Simplex Virus Type 1

HSV-2 – Herpes Simplex Virus Type 2

IFN-α – Interferon Alpha

FN-β – Interferon Beta

IFN-γ – Interferon Gamma

IL – Interleukin

IL-18R – Interleukin 18 Receptor

ISGs – Interferon-Stimulated Genes

KLRG1 – Killer Cell Lectin-Like Receptor G1

LAT – Latency-Associated Transcript

MHC – Major Histocompatibility Complex

mRNA – Messenger RNA

NF-κB – Nuclear factor kappa B

NK – Natural Killer

PD-1 - Programmed Cell Death Protein 1

PRRs – Pattern Recognition Receptors

RIG-I – Retinoic Acid-Inducible Gene I

RNA - Ribonucleic Acid

RUNX3 – Runt-Related Transcription Factor 3

S1P – Sphingosine-1-Phosphate

S1PR1 – Sphingosine-1-Phosphate Receptor 1

STING – Stimulator of Interferon Genes

T-BET - T-box expressed in T cells

TCF-1 -T cell factor 1

TCM – Central Memory T Cell

TCR – T Cell Receptor

TEM – Effector Memory T Cell

TGF-β – Transforming Growth Factor Beta

TLR – Toll-Like Receptor

TNF-α – Tumor Necrosis Factor Alpha

TRM – Tissue-Resident Memory T Cell

UV – Ultravioleta

VZV - Varicella-Zoster Virus

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# 1. REFERENCIAL TEÓRICO

## 1.1 Panorama da infecção por Herpes Simplex Virus

Os Vírus Herpes Simples 1 (HSV-1) e 2 (HSV-2) ou, *Simplexvirus humanalpha1* e *Simplexvirus humanalpha2*, pertencem à família *Herpesviridae* e gênero *Simplexvirus*. Esses vírus apresentam envelope lipídico como revestimento externo, no qual estão ancoradas glicoproteínas que interagem com diferentes receptores celulares, mediando a entrada viral, assim, infectam diferentes espécies, incluindo humanos e modelos animais em pesquisa.[1]

Após a infecção primária, os HSVs estabelecem latência em neurônios sensoriais localizados em gânglios próximos ao sítio de infecção inicial. Durante esse estado, o genoma viral persiste na forma de episssomas com expressão gênica restrita, caracterizada pela ausência de expressão dos genes líticos e pela transcrição limitada a poucos microRNAs virais, como o transcrito associado à latência (LAT), sem produção de novas partículas virais.[2] Episódios de reativação podem ocorrer ao longo da vida do hospedeiro, levando à replicação viral e migração anterógrada até os tecidos periféricos, onde se manifestam clinicamente. As lesões recorrentes são mais frequentemente observadas nas regiões orofacial e genital. Em casos menos frequentes, a infecção pode evoluir para complicações graves, como encefalite herpética, hepatite ou infecções oculares, que podem resultar em perda da visão.[3]

Em 2016, estimou-se que mais de 3,5 bilhões de indivíduos no mundo apresentem soropositividade para HSV-1 e centenas de milhões para HSV-2, sendo que uma parcela significativa das infecções é assintomática.[4] Apesar da

ausência de sintomas em muitos casos, a transmissão viral pode ocorrer, contribuindo para a ampla disseminação desses patógenos na população. O HSV-1 é predominantemente transmitido por contato oral, estando associado principalmente a lesões orofaciais, enquanto o HSV-2 é mais frequentemente transmitido por via sexual e associado a infecções genitais.[3] A prevalência de soropositividade para HSV-2 apresenta variação de acordo com fatores demográficos, comportamentais e geográficos, incluindo idade, sexo, práticas sexuais e acesso a serviços de saúde. De modo geral, observa-se aumento da soroprevalência com a idade, refletindo a exposição cumulativa ao vírus. Regiões com maior incidência de infecções sexualmente transmissíveis e menor acesso a medidas de prevenção e diagnóstico também tendem a apresentar maiores taxas de infecção.[5–7] Diante de sua alta prevalência, capacidade de persistência no hospedeiro e potencial para causar complicações, o HSV-1 e HSV-2 configuram importantes problemas de saúde pública no Brasil e no mundo.

A transmissão pode ocorrer mesmo na ausência de lesões visíveis, devido à eliminação viral assintomática, o que representa um desafio adicional para o controle da infecção. Compreender a epidemiologia local e global é crucial para a criação de estratégias eficazes de prevenção e controle. Nesse contexto, medidas preventivas, como o uso de preservativos e estratégias de educação em saúde, desempenham papel fundamental na redução da transmissão.[8] Adicionalmente, a otimização do protocolo de tratamento farmacológico constitui um componente estratégico no manejo da infecção, visando não apenas à redução da duração e da gravidade dos episódios clínicos, mas também à diminuição viral e, consequentemente, do risco de transmissão. Paralelamente,

a modulação da resposta imune do hospedeiro emerge como um eixo promissor, uma vez que o fortalecimento da imunidade antiviral pode contribuir tanto para o controle da replicação viral quanto para a redução da frequência de reativações.[9] Nesse cenário, abordagens integradas que combinem intervenções farmacológicas e estratégias imunomoduladoras despontam como alternativas promissoras para o aprimoramento do manejo clínico e do controle da disseminação viral.

### 1.2 Aspectos Estruturais e Funcionais da Infecção pelo HSV-2

A infecção por HSV-2 envolve uma série de processos coordenados que permitem sua entrada, replicação e disseminação no hospedeiro. Estruturalmente, o virion é composto por três elementos principais: um nucleocapsídeo icosaédrico que abriga o genoma de DNA fita dupla linear, uma camada de tegumento contendo proteínas multifuncionais, e um envelope lipídico externo no qual estão inseridas glicoproteínas virais, incluindo gB, gC, gD, gH e gL.[10]

O processo de entrada do vírus é mediado por interações específicas entre glicoproteínas do envelope e receptores celulares. Inicialmente, as proteínas gC e gD se ligam a receptores celulares, como o sulfato de heparano presente na superfície celular, promovendo a aproximação do vírus à membrana plasmática. Em seguida, a glicoproteína gD interage com receptores de entrada mais específicos, desencadeando alterações conformacionais que ativam o complexo gH/gL e, subsequentemente, gB, culminando na fusão entre o envelope viral e a membrana celular. Esse processo resulta na liberação do nucleocapsídeo e das proteínas do tegumento no citoplasma.[11]

Após a entrada, o nucleocapsídeo é transportado até o núcleo, onde o DNA viral é liberado. A expressão gênica ocorre de maneira temporalmente regulada, iniciando-se com os genes imediatos precoces (IE), que codificam proteínas regulatórias responsáveis por ativar a transcrição de genes precoces (E). Estes, por sua vez, estão envolvidos principalmente na replicação do DNA viral. Por fim, os genes tardios (L) codificam proteínas estruturais necessárias para a montagem de novos vírions.[12]

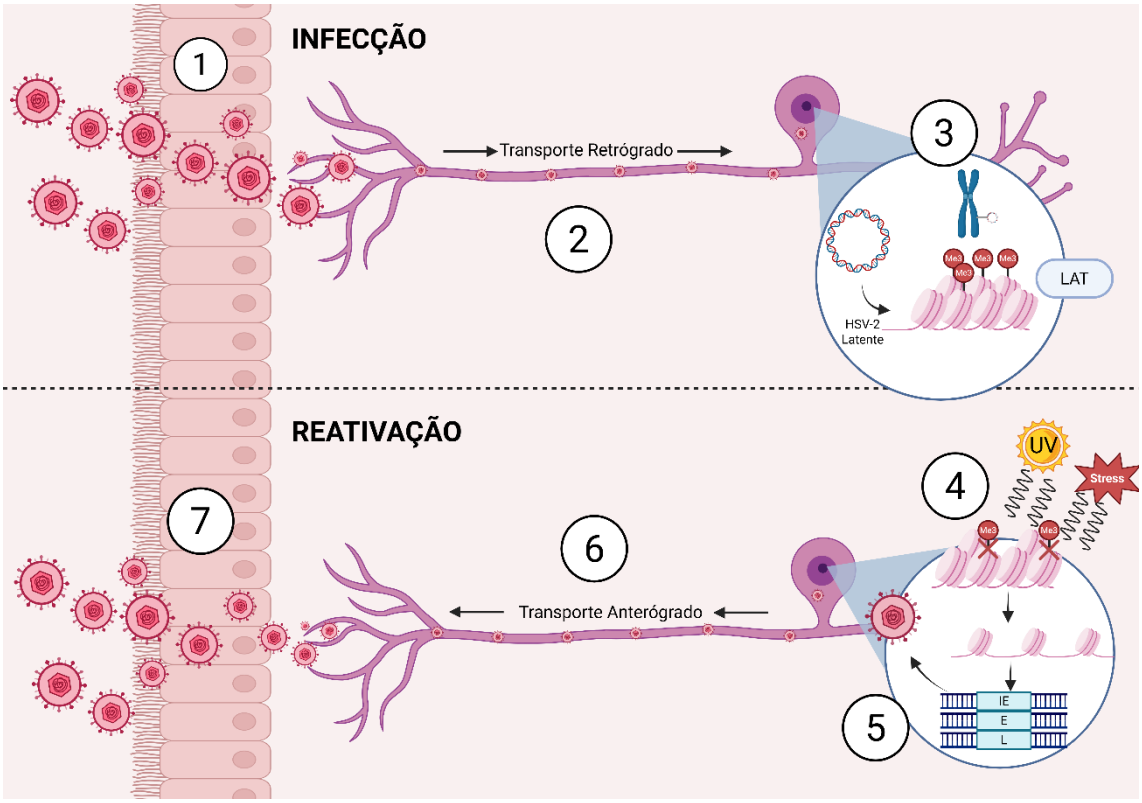
A montagem do virion ocorre no núcleo, seguida de processos de aquisição de envelope e maturação, que envolvem múltiplas etapas de brotamento e remodelação de membranas intracelulares. As partículas virais maduras são então transportadas e liberadas da célula hospedeira, permitindo a infecção de células adjacentes e a disseminação local do vírus.[12,13]

Após a infecção primária na mucosa, o HSV-2 invade terminações nervosas sensoriais e migra de forma retrógrada até os gânglios nervosos, onde estabelece latência (Figura 1). Nesse estado, o genoma viral persiste no núcleo dos neurônios como episssoma, com expressão gênica limitada principalmente à transcrição de microRNAs associado à latência (LAT), sem produção de partículas virais.[14,15] O genoma do HSV associa-se às histonas do hospedeiro, organizando-se em uma estrutura semelhante à cromatina. Nesse estado, a expressão dos genes virais é amplamente reprimida por modificações epigenéticas, especialmente a metilação de histonas em regiões promotoras de genes líticos, o que mantém o genoma em um perfil transcricionalmente inativo, enquanto regiões associadas à latência permanecem mais acessíveis. A reativação viral envolve a remodelação dessa cromatina, com a remoção dessas marcas repressoras, incluindo processos de desmetilação, e a aquisição de

modificações associadas à ativação transcricional, permitindo a reexpressão dos genes virais e o retorno ao ciclo replicativo.[16]

Esse processo pode ser desencadeado por diversos estímulos, como estresse, imunossupressão ou alterações inflamatórias locais, levando à reexpressão de genes líticos, replicação viral e transporte anterógrado do vírus de volta ao tecido periférico. Como consequência, ocorrem episódios recorrentes de infecção na mucosa genital, frequentemente no mesmo local da infecção inicial.[17]

**Figura 1: Modelo de infecção e reativação local dos HSVs**




Legenda: (1) O HSV-2 Infecta o hospedeiro pelas células epiteliais da mucosa; (2) O vírus atinge as terminações nervosas dos neurônios sensoriais presentes no sítio de infecção e se desloca por dentro do neurônio por transporte retrógrado; (3) Ao atingir os gânglios, após o vírus atingir o núcleo, o vírus não se replica, mas o DNA viral se circunda, estabelecendo-se na forma de episssoma associado ao DNA do hospedeiro. Esse episssoma fica conjugado às histonas do hospedeiro, sendo assim restrito por processos epigenéticos como metilações e acetilações das histonas, sendo apenas transcritos associados a latência (LAT) sendo transcritos; (4) Processos como estresse fisiológico ou dano físico como luz UV podem desfazer essas

metilações e acetilações das histonas, expondo regiões codificantes dos genes líticos do vírus; (5) O vírus retorna ao ciclo replicativo com a formação de novos virions; (6) As partículas virais fazem o transporte anterógrado de volta para o tecido; (7) Ocorre a reinfecção de células epiteliais da mucosa com recidiva dos sintomas. Autor: Alisson Felipe Haubert

Além de sustentar a persistência viral e os episódios recorrentes de infecção, a capacidade do HSV-2 de alternar entre os estados lítico e latente reflete uma complexa interação entre o vírus e o sistema imunológico do hospedeiro. Durante todas as fases da infecção, desde a invasão inicial da mucosa até o estabelecimento da latência e os eventos de reativação, diferentes componentes da imunidade inata e adaptativa atuam na tentativa de conter a replicação viral e limitar a disseminação do vírus. Entretanto, o HSV-2 desenvolveu múltiplos mecanismos de evasão imunológica que favorecem sua permanência no organismo e dificultam a eliminação completa da infecção. Nesse contexto, compreender a resposta imune ao HSV-2 é fundamental para elucidar os mecanismos envolvidos no controle da infecção, na recorrência clínica e no desenvolvimento de estratégias terapêuticas e vacinais mais eficazes.

### 1.3 Resposta imunológica ao HSV-2

A resposta imunológica ao HSV-2 envolve a atuação coordenada de mecanismos da imunidade inata e adaptativa, essenciais para o controle da infecção, limitação da disseminação viral e estabelecimento de memória imunológica. Inicialmente, a detecção do vírus é mediada por receptores de reconhecimento de padrão (PRRs), responsáveis por identificar ácidos nucleicos virais. Entre esses, destaca-se o TLR9, localizado em endossomos, que reconhece DNA viral contendo sequências CpG não metiladas (regiões em que

citosina e guanina estão adjacentes), reconhecidas como padrões moleculares associados a patógenos.[18] No citosol, sensores como o cGAS detectam DNA de dupla fita e ativam a via STING.[19] Além disso, receptores semelhantes ao RIG-I podem ser ativados por espécies de RNA geradas durante a replicação viral, incluindo intermediários de RNA de fita dupla.[20]

A ativação desses sensores desencadeia cascatas de sinalização que culminam na produção de interferons do tipo I (IFN-α e IFN-β), além de citocinas pró-inflamatórias como TFN-α e IL-1β.[21] Esses interferons atuam de forma autócrina e parácrina, induzindo a expressão de genes estimulados por interferon (ISGs), os quais estabelecem um estado antiviral que é caracterizado pela inibição da replicação viral, degradação de ácidos nucleicos virais, bloqueio da síntese proteica e aumento da apresentação de antígenos via MHC. Esses mecanismos são fundamentais para conter a infecção nas fases iniciais e orientar o desenvolvimento da resposta imune adaptativa.[22]

Diversas células da imunidade inata participam ativamente nesse processo, incluindo monócitos, neutrófilos, macrófagos, células dendríticas (DCs) e células *natural killer* (NK). Essas células atuam tanto na contenção inicial da infecção quanto na modulação da resposta adaptativa subsequente, por meio da produção de citocinas, atividade citotóxica e apresentação de antígenos.[23–25]

As células dendríticas, durante a infecção por HSV, são recrutadas para o local da lesão, invadem a junção derme-epidérmica, capturam antígenos virais e sofrem ativação por meio de receptores como TLR2 e TLR9. A ativação dessas vias leva à indução de fatores de transcrição, como NF-κB, promovendo a produção de citocinas pró-inflamatórias, incluindo TNF-α, IL-1β e IL-6, bem como quimiocinas como CCL2 e CXCL10, que contribuem para o recrutamento celular

e a maturação das DC's.[18] Subpopulações especializadas de células dendríticas, como as DCs CD103$^+$ (integrina αE), desempenham papel importante na imunidade contra o HSV-2 por sua capacidade de apresentação cruzada de antígenos virais. Após a infecção viral na mucosa genital, essas células capturam antígenos no tecido e migram para os linfonodos drenantes, onde ativam linfócitos T CD8$^+$ virgens via MHC classe I. Esse processo é essencial para a geração de respostas citotóxicas específicas e influencia a capacidade desses linfócitos de retornar ao tecido e adquirir características associadas à retenção tecidual.[26] A comunicação entre respostas inata e adaptativa ocorre por meio das células apresentadoras de antígeno fagocíticas, sendo essencial para a ativação de linfócitos T CD4$^+$ e CD8$^+$ e para o controle da replicação viral. Além disso, essas respostas desempenham papel central na contenção dos episódios de reativação do herpes, nos quais o vírus latente nos gânglios sensoriais retoma a expressão de genes líticos e retorna ao tecido periférico, sendo rapidamente reconhecido e controlado pela imunidade local estabelecida na mucosa.[27]

Os linfócitos T CD4$^+$ desempenham papel crucial no sistema imunológico adaptativo, orquestrando respostas a infecções e contribuindo para a sobrevivência do hospedeiro.[28] Pesquisas em modelo murino de infecção por HSV-1 demonstram a importância das células T CD4$^+$ na prevenção a recidiva e resolução da lesão genital gerada pelos HSVs de doenças genitais.[29] No modelo murino de infecção por HSV-2, após inoculação intravaginal, células T CD4$^+$ podem persistir em tecidos neurais, como a medula espinhal e os gânglios da raiz dorsal. A manutenção dessas células nesses sítios parece depender de sinais locais de sobrevivência e de níveis baixos ou intermitentes de

apresentação antigênica via MHC classe II, possivelmente associados a eventos subclínicos de reativação viral. Além disso, parte dessas células pode adquirir características de retenção tecidual, contribuindo para o controle da replicação viral tanto em compartimentos neurais quanto no tecido periférico após a infecção primária.[30–32]

Em infecções por patógenos intracelulares, os linfócitos T $CD8^+$ desempenham função essencial no combate aos microrganismos.[33] A diferenciação dessas células em linfócitos T citotóxicos (CTLs) é regulada por sinais provenientes da ativação do receptor de célula T (TCR), coestimulação e citocinas inflamatórias, como IL-12 e interferons do tipo I, que induzem a expressão de mediadores de citotoxicidade, como perforina e granzimas, o que permite a sua ação protetora em tecidos não-linfoides infectados por patógenos intracelulares, principalmente vírus.[34] No contexto de uma infecção viral, o IFN-γ secretado por linfócitos T $CD8^+$ atua na célula apresentadora o que aumenta a expressão de MHC de classe I associado a peptídeos virais, esse processo colabora para a atividade antiviral.[35]

Em resposta específica ao HSV, linfócitos T $CD8^+$ são cruciais especialmente no controle da latência e recidivas. Essas células atuam tanto por mecanismos citotóxicos, eliminando células infectadas por meio da liberação de perforina e granzimas, quanto por mecanismos não citolíticos, como a produção de citocinas antivirais, especialmente IFN-γ.[36] No contexto da latência, linfócitos T $CD8^+$ presentes nos gânglios sensoriais contribuem para a manutenção do vírus em estado latente ao suprimir a reativação viral, inibindo a expressão de genes líticos sem necessariamente induzir a morte dos neurônios infectados.

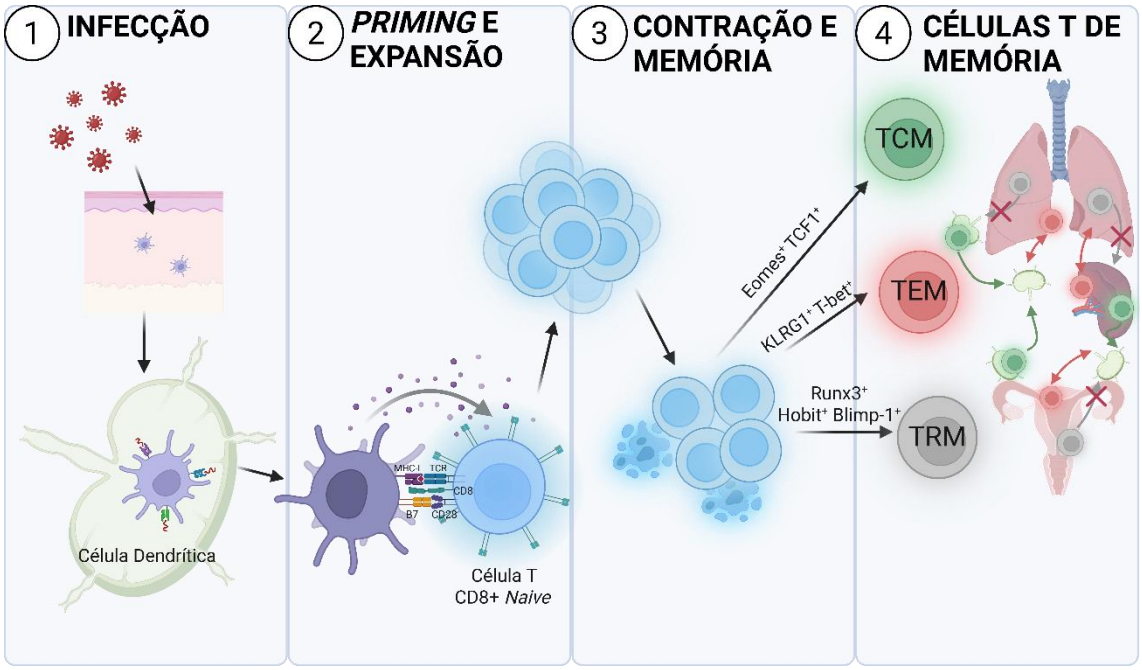
Durante episódios de reativação, essas células também atuam no tecido periférico, limitando a replicação viral e a extensão da lesão.[27,37]

Entretanto, o HSV utiliza uma variedade de estratégias para evadir e modular a resposta imune do hospedeiro e favorecer a sua própria replicação e disseminação. Isso inclui a capacidade de suprimir a expressão de genes envolvidos na resposta antiviral do hospedeiro, como por exemplo a manipulação da função da proteína STING (Stimulator of Interferon Genes) durante sua replicação. A STING é uma proteína importante na resposta imunológica antiviral, pois desencadeia a produção de interferons, que são proteínas essenciais na defesa do organismo contra infecções virais com a redução da apresentação de antígenos via MHC, impedindo assim a ativação efetiva das células imunes.[19] O HSV também possui mecanismos para evitar a detecção pelo sistema imunológico, como a evasão da apresentação de antígenos e a inibição da ação das células imunes efetoras, como as células NK e as células T $CD8^+$. [38,39]

Classicamente, após a fase efetora da resposta imune, aproximadamente 90-95% das células T $CD8^+$ efetoras sofrem apoptose, permanecendo um contingente de 5-10% que dá origem às populações de memória.[40] Essas células podem ser classificadas em subpopulações com características fenotípicas e funcionais distintas, incluindo células T de memória central (TCM), memória efetora (TEM) e células T residentes de tecido (TRM). A diferenciação dessas populações é regulada por programas transcricionais específicos, conforme ilustrado na Figura 2. Estudos em infecções por herpesvírus demonstram que a formação de células T de memória é essencial para o controle da latência viral e da recorrência da infecção.[41]

As células TCM permanecem principalmente em órgãos linfoides secundários, apresentam alta capacidade proliferativa e expressam marcadores como CD62L e CCR7. Esse perfil está associado à expressão de fatores de transcrição como TCF-1 (T cell factor 1) e Eomes (Eomesodermin), relacionados à manutenção de um estado menos diferenciado e à autorrenovação celular.[42] Em modelos de infecção por HSV, células com características de TCM foram associadas à manutenção prolongada da resposta antiviral e à rápida expansão após reexposição ao antígeno.[43] Em contraste, as células TEM circulam entre o sangue e tecidos periféricos, apresentando funções efetoras rápidas e maior grau de diferenciação, associado à expressão de moléculas como T-bet (T-box expressed in T cells) e KLRG1 (Killer cell lectin-like receptor subfamily G member 1).[44] As células TRM constituem uma população especializada que permanece de forma estável em tecidos não linfoides, como a mucosa genital.[45] Essas células são tipicamente caracterizadas pela expressão de marcadores como CD69 e CD103, além de apresentarem baixa expressão de moléculas associadas à recirculação, como CCR7 e CD62L.[31,32,45] Durante infecções herpéticas, essas células contribuem para respostas citotóxicas imediatas em sítios periféricos de infecção e reativação viral.[32] As características fenotípicas, mecanismos de diferenciação e funções dessas células serão abordados no próximo tópico.

**Figura 2 – Esquema da geração de linfócitos T CD8⁺ de memória durante uma infecção viral**




Legenda: (1) Em uma infecção viral de pele ou mucosa, partículas virais são capturadas e processadas por células dendríticas (DCs) presentes no tecido, que migram para os linfonodos drenantes. (2) As DCs apresentam epítopos virais a linfócitos T CD4⁺, sendo licenciadas por essas células, e posteriormente, ou mesmo concomitantemente, apresentam antígenos virais a linfócitos T CD8⁺ virgens específicas, juntamente com sinais coestimulatórios e citocinas, induzindo ativação e expansão clonal. Mecanismos de crosspresentration também podem participar dessa etapa (3) Após a resolução da infecção, grande parte das células T efetoras sofre apoptose devido à redução dos sinais de proliferação e sobrevivência, permanecendo apenas uma fração de células de memória. (4) A diferenciação dos linfócitos T CD8⁺ de memória depende dos sinais recebidos durante o *priming*, da ação de fatores de transcrição e do microambiente tecidual, originando subpopulações de memória central (TCM), memória efetora (TEM) e memória residente em tecido (TRM). Autor: Alisson Felipe Haubert

### 1.4 Linfócitos T residentes de memória

Ha cerca de uma década, as células TRM foram identificadas como um novo subgrupo de células T de memória, reformulando a antiga classificação que dividia as células de memória em efetoras e centrais.[46] Inicialmente, acreditava-se que as células T migravam para os tecidos durante a infecção, saindo após a

eliminação do patógeno ou passando por apoptose.[47] No entanto, como a evolução dos estudos, descobriu-se que linfócitos T CD8+ específicos permanecem nos tecidos não linfoides por períodos prolongados após infecção viral [48,49]. As primeiras evidências de células T de memória residentes em tecidos surgiram em modelos de infecção respiratória por influenza, nos quais foi observado que linfócitos T específicos permaneciam no pulmão e nas vias aéreas após a resolução da infecção.[50] Esses achados forneceram a base para a posterior definição das células T residentes de tecido (TRM) como uma subpopulação distinta de memória imunológica e desde então, foram identificadas em vários tecidos não linfoides, estando presentes praticamente em todos os órgãos incluindo pele e mucosas.[31,32,45] Embora as células T CD4+ e CD8+ possam colonizar tecidos, as células T CD8+ foram identificadas como o principal grupo que exibe residência nesses locais.[51]

As TRMs demonstram capacidade de imunovigilância, podendo se expandir localmente e liberar citocinas imunomoduladoras como IFN-γ e TNF-α, além de quimiocinas como CXCL9 e CXCL10, que promovem mecanismos antivirais no tecido e o recrutamento de outras células do sistema imunológico, que contribuem para a contenção da disseminação da infecção. Posteriormente, as TRMs combatem diretamente as células infectadas que expressam o antígeno cognato.[52–55] As TRMs que se estabelecem na pele, têm a capacidade de estender dendritos para patrulhar os arredores de forma a interagir com microambiante local e encontrar APCs. Após o encontro com APCs que apresentam o antígeno cognato a TRM específica retorna ao formato arredondado após a ativação.[56]

A diferenciação das TRMs é regulada por sinais do microambiente tecidual local e outras vias, incluindo citocinas, interação com o tecido e a intensidade da ativação via TCR. O reconhecimento do antígeno pelo TCR é fundamental para a formação e estabilidade das TRMs, sendo que a afinidade do TCR com o antígeno influencia essa formação de modo diferente dependendo do tecido, afinidades altas favorecem TRMs no cérebro e rim, enquanto afinidades mais baixas ou intermediárias podem favorecer TRMs nos pulmões. Estímulos subótimos podem, em alguns casos, recrutar mais TRMs funcionais, indicando que sinais mais intensos nem sempre são melhores para a residência.[57–59]

A regulação das TRMs por citocinas e quimiocinas é fundamental para sua diferenciação e manutenção nos tecidos. O TGF-β desempenha papel central na indução do fenótipo residente, especialmente por promover a expressão de CD103, marcador associado à retenção em epitélios. A interleucina-15 (IL-15) é essencial para a sobrevivência e proliferação homeostática dessas células em diferentes tecidos. Ademais, quimiocinas e seus receptores, como CXCR6 e CXCR3, por meio da interação com ligantes como CXCL16 e CXCL9/10, contribuem para o recrutamento, posicionamento e organização espacial das células T nos tecidos periféricos.[60–62]

Além disso, a IL-10 tem sido associada à modulação do microambiente inflamatório local, contribuindo para limitar dano tecidual excessivo e favorecer a manutenção funcional de células residentes durante respostas secundárias.[63] Já a IL-18, estudos indicam que a redução da expressão de seu receptor (IL-18R) acompanha o processo de diferenciação de células TRM em determinados tecidos, estando associada ao estabelecimento da residência tecidual e à

distinção entre populações residentes e circulantes.[64] Esses achados reforçam que a formação de células TRM depende de múltiplos sinais locais e de mudanças fenotípicas progressivas ao longo da resposta imune.

A programação transcricional das TRMs é regulada por uma rede de fatores que promovem a residência tecidual e suprimem a recirculação. O fator de transcrição Runt-related transcription factor 3 (RUNX3) atua como um regulador central da diferenciação de células TRM, promovendo a expressão de moléculas associadas à residência tecidual, como CD69 e CD103, enquanto reprime genes envolvidos na migração e saída celular dos tecidos, como S1pr (receptor 1 de esfingosina-1-fosfato).[65] De forma complementar, Homolog of Blimp-1 in T cells (Hobit) e B lymphocyte-induced maturation protein-1 (Blimp-1), contribuem para a repressão de programas gênicos associados à circulação, favorecendo a aquisição e manutenção do fenótipo residente.[66] A redução na expressão de T-bet (T-box expressed in T cells) e Eomes (Eomesodermin) também é importante para a transição para um estado mais quiescente, compatível com a permanência prolongada no tecido.[67] Além disso, o fator de transcrição Bhlhe40 Basic helix-loop-helix family member e40 (Bhlhe40) participa da regulação metabólica dessas células, promovendo adaptação funcional e suporte à sua persistência em ambientes teciduais, principalmente pulmonares e especialmente sob condições inflamatórias.[68]

Uma vez estabelecidas nos tecidos, as células TRM mantêm um perfil transcricional compatível com rápida resposta efetora, podendo expressar RNAs mensageiros (mRNAs) relacionados a mediadores citotóxicos, como a granzima B, particularmente após estímulos locais que desencadeiam sua reativação funcional.[69] A produção das citocinas IFN-γ, TNF-α e IL-2 por essas células

desempenha um papel crucial na contenção da infecção local e na manutenção da frequência de TRMs nos tecidos. Quando secretadas, essas citocinas efetoras estimulam a maturação das células dendríticas, aumentam a capacidade citotóxica das células natural killer e reforçam a resistência do tecido à infecção. O IFN-γ também promove a amplificação da resposta inflamatória local por meio da indução de quimiocinas, especialmente CXCL9 e CXCL10, bem como pela maior expressão de moléculas de adesão endotelial, facilitando o recrutamento e a migração de células imunes para o tecido infecta.[70] As células TRM, ao produzirem rapidamente citocinas inflamatórias, desempenham um papel crucial na contenção precoce da infecção. Adicionalmente, a expressão de receptores inibitórios, como o PD-1, atua na modulação da atividade dos linfócitos T no tecido, reduzindo sinais de ativação excessiva. Essa regulação é particularmente importante em ambientes sensíveis, como mucosas, onde uma resposta imune exacerbada pode causar dano tecidual. Dessa forma, o PD-1 contribui para o equilíbrio entre controle da infecção e preservação da integridade do tecido.[71,72]

A retenção tecidual das células TRM é assegurada por dois principais mecanismos: adesão ao tecido local e resistência a sinais que poderiam estimular seu retorno ao sangue periférico ou a tecidos linfoides. As TRMs exibem pouca responsividade à esfingosina-1-fosfato (S1P), secretada por células endoteliais, devido à expressão de CD69, uma lectina do tipo C, que suprime a expressão de receptores de S1P.[73] Adicionalmente, as células TRM caracterizam-se pela ausência de expressão de CD62L (L-selectina) e CCR7, moléculas associadas ao homing linfonodal e à recirculação por tecidos linfoides secundários, reforçando seu perfil de residência tecidual.[74] A manutenção da

residência tecidual também depende da expressão da integrina CD49a, cuja interação com componentes da matriz extracelular, particularmente colágeno IV e laminina, contribui para a permanência das células TRM nos tecidos não linfoide.[69]

As células TRM ainda compartilham semelhanças com as células T efetoras e de memória incluindo a expressão de CD44, importante para a interação com o ácido hialurônico da matriz extracelular. Adicionalmente, moléculas associadas à residência tecidual, como CD103, contribuem para o direcionamento e retenção dessas células em epitélios por meio de sua interação com a E-caderina.[74,75] Essas características permitem o estabelecimento duradouro das células TRM em tecidos de barreira, onde exercem funções de vigilância imunológica e promovem resposta rápida contra infecções virais.

Em conjunto, essas características fenotípicas, funcionais e moleculares demonstram que as células TRM constituem uma população altamente especializada de linfócitos T de memória, adaptada à permanência prolongada em tecidos não linfoides. A expressão de moléculas de adesão, receptores associados à retenção tecidual e fatores de transcrição específicos permite que essas células estabeleçam nichos locais de vigilância imunológica, promovendo resposta rápida frente à reexposição antigênica. Em infecções virais de mucosa, particularmente aquelas causadas pelo HSV-2, as células TRM desempenham papel fundamental no controle local da replicação viral e na contenção da disseminação do vírus, destacando sua relevância na imunidade antiviral em tecidos genitais. Nesse contexto, compreender os mecanismos envolvidos na geração, manutenção e função das células TRM durante a infecção genital pelo

HSV-2 é essencial para o desenvolvimento de estratégias terapêuticas e vacinais mais eficazes.

### 1.5 TRMs CD8+ na infecção genital por HSV-2

Os tecidos de barreira constituem a primeira linha de defesa contra patógenos, apresentando uma organização imunológica altamente especializada. Um dos principais tecidos que desempenha papel crucial como barreira contra patógenos infecciosos é a pele. A epiderme abriga principalmente células TRM $CD8^+$ na população de células T de memória, e em resposta à exposição a patógenos seu número aumenta rapidamente[76]. De maneira semelhante, outro órgão que mantém contato direto com patógenos externos é a mucosa genital feminina, que é composta por duas regiões principais: o endométrio e a endocérvice, que compõem a porção superior do sistema genital feminino, enquanto a vagina e a ectocérvix constituem a porção inferior. A mucosa genital abriga uma variedade de células do sistema imunológico, destacando-se as células T de memória.[77]

Os linfócitos TRM $CD8^+$ não se dispersam de maneira uniforme nos tecidos de risco ao longo do tempo, na verdade eles persistem em uma região específica após o reconhecimento de um antígeno. Um estudo que empregou modelos matemáticos para analisar a localização desses linfócitos TRM gerados durante uma infecção por HSV-2 mostrou que a posição dessas células é espacialmente delimitada na mucosa, correlacionando-se com o ponto de recidiva e sendo pouco distribuídas no tecido adjacente local.[78] Essa organização espacial pode influenciar diretamente a eficiência do controle viral durante episódios de recidiva, uma vez que a presença localizada de células

TRM permite o reconhecimento mais rápido de células infectadas e a contenção precoce da replicação viral no sítio de reativação. Em contrapartida, regiões com menor densidade dessas células podem apresentar controle menos eficiente, favorecendo a disseminação local do vírus.[77,79]

Apesar da resposta imunológica coordenada contra o HSV, o vírus consegue estabelecer a latência nos nervos do hospedeiro, persistindo ao longo da vida. Em determinadas circunstâncias, influenciadas por fatores ambientais, bioquímicos ou imunológicos, ocorre a recidiva do vírus na mesma região da infecção primária.[80] Frequentemente, diante desse quadro, muitos optam por intervenções farmacológicas antivirais, sendo o aciclovir o medicamento mais comumente utilizado, seguido por seus derivados, como valaciclovir, para reduzir a replicação viral e controlar os sintomas clínicos.[81] No entanto, o uso indiscriminado de prescrição do aciclovir e similares e a automedicação tem contribuído para preocupações relacionadas ao surgimento de cepas resistentes a essas drogas.[82]

Em contextos clínicos específicos, como em pacientes submetidos a transplantes de órgãos ou terapias com células CAR-T, o uso de aciclovir como profilaxia antiviral é amplamente estabelecido devido ao alto risco de reativação de herpesvírus associado à disfunção imunológica. Nesses cenários, a administração profilática reduz de forma eficaz a replicação viral e a incidência de infecções por HSV e VZV.[83–86]

No entanto, do ponto de vista imunológico, a disponibilidade de antígeno viral desempenha papel central na ativação, expansão e diferenciação de linfócitos T, incluindo a geração de células com características de residência tecidual. A intensidade e a duração da exposição antigênica, aliadas ao contexto

inflamatório local, são determinantes para o estabelecimento de respostas T efetoras e para a aquisição de fenótipos associados à retenção no tecido.[57–59]

Dessa forma, intervenções que reduzem precocemente a carga viral, como o uso de aciclovir, podem impactar esses processos ao limitar a disponibilidade de antígeno no tecido, influenciando a magnitude e o perfil da resposta imune local. Assim, os efeitos observados não decorrem diretamente do fármaco, mas da modulação do estímulo antigênico que orienta a resposta imune durante a fase aguda da infecção.[58]

No contexto das células TRM, a dinâmica dessas células está intimamente relacionada ao microambiente tecidual e à disponibilidade de antígeno, fatores que influenciam sua geração, manutenção e função. Durante a fase aguda da infecção, a persistência do estímulo antigênico local pode influenciar a ativação, expansão e diferenciação de linfócitos T $CD8^+$, favorecendo o estabelecimento de populações com características de residência tecidual. Além disso, evidências indicam que a manutenção de células TRM em determinados contextos pode depender da exposição contínua ou intermitente a antígenos locais, especialmente em infecções persistentes ou recorrentes, como as causadas por herpesvírus. Dessa forma, alterações na carga viral durante a infecção inicial podem impactar diretamente os sinais necessários para a programação e o estabelecimento dessas células no tecido.[59]

Diante disso, embora estudos prévios indiquem o papel protetor das células TRM na infecção genital por HSV-2, aspectos relacionados à geração dessas células na primo-infecção permanecem pouco caracterizados. Além disso, considerando o amplo uso do aciclovir na prática clínica e seu efeito direto sobre a replicação viral, torna-se relevante investigar se essa intervenção

terapêutica pode influenciar a frequência e a manutenção das TRMs na mucosa genital.

Nesse sentido, a investigação da resposta imune mediada por células TRM em infecção por HSV-2, bem como a avaliação do impacto da terapia antiviral sobre essa população, pode contribuir para o avanço do conhecimento sobre a imunidade tecidual e para o desenvolvimento de estratégias terapêuticas mais eficazes, que conciliem o controle viral com a preservação da imunidade de longo prazo.

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# 3. OBJETIVOS

## 3.1 Objetivo Geral:

Caracterizar os precursores de linfócitos T $CD8^+$ TRMs durante a fase aguda da infecção pelo HSV-2, avaliando como o controle da carga antigênica mediado pelo tratamento com aciclovir influencia sua geração e estabelecimento no tecido genital em modelo animal.

## 3.2 Objetivos Específicos:

- ✓ Determinar a frequência e distribuição tecidual das subpopulações de linfócitos T residentes de memória presentes na mucosa genital na infecção por HSV-2.
- ✓ Investigar se a dose de inóculo viral influencia na composição e distribuição dos linfócitos T residentes de memória.
- ✓ Investigar se o controle do antígeno pelo tratamento com aciclovir influencia a população, distribuição e atividade funcional dos linfócitos T residentes de memória na mucosa genital.

## 4. ARTIGO CIENTÍFICO REDIGIDO EM INGLÊS

# Acyclovir-Mediated Reduction of Antigen Load Limits Early CD8$^{+}$ TRM Precursor Formation During Genital HSV-2 Infection

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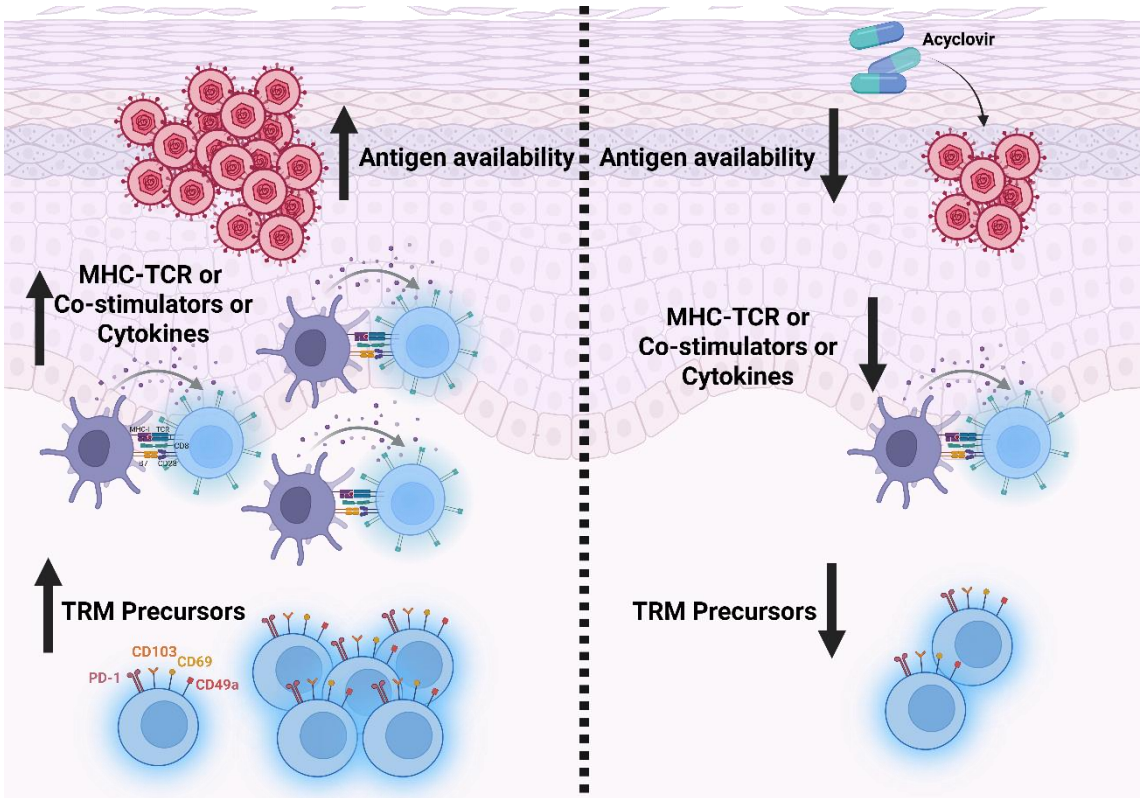
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## Graphical Abstract



## Abstract

Herpes simplex virus 2 (HSV-2) establishes persistent infection in the genital mucosa and induces local immune responses that are critical for viral control. Tissue-resident memory T cells (TRM) are important mediators of local protection, but the influence of antigen availability on their early differentiation remains incompletely understood. This study investigated the effects of initial viral load and antiviral treatment on the generation of $CD8^+$ T cell populations associated with tissue residency during acute HSV-2 infection. Female C57BL/6 mice were infected intravaginally with increasing doses of HSV-2 or treated with acyclovir following infection. Fourteen days post-infection, genital tissue, draining lymph nodes, and spleens were analysed by flow cytometry. Higher viral inocula resulted in increased disease severity and promoted the accumulation of $CD8^+$ T cells expressing activation markers and phenotypic characteristics associated with TRM precursors in the genital mucosa. In contrast, acyclovir treatment reduced lesion severity and mortality and was associated with decreased frequencies of activated $CD8^+$ T cells and TRM precursor populations in the infected tissue. These effects were largely restricted to the local compartment, with limited impact on systemic immune responses. Together, these findings indicate that antigen availability during acute infection is a key determinant of local $CD8^+$ T cell activation and early tissue-resident memory programming.



## Keywords

HSV-2; Tissue-resident memory T cells; CD8 T cells; Acyclovir; Viral load; Genital mucosa.

## Introduction

Herpes simplex virus 2 (HSV-2) is a globally widespread pathogen that establishes persistent infection in the genital tissue, characterized by recurrent episodes of viral reactivation following the establishment of latency in sensory neurons[1, 2]. During latency, the viral genome persists as an episome with restricted gene expression, primarily limited to latency-associated transcripts (LAT), without production of infectious virions. Reactivation can be triggered by physiological stress or immunosuppression, leading to renewed viral replication and transport back to peripheral tissues [3, 4]. This recurrent nature of HSV-2 infection highlights the critical role of local immune responses in controlling viral replication at barrier sites, particularly within the genital mucosa, where primary pathogen sensing and antiviral immune activation occur [5]. The genital mucosa represents a highly specialized immunological niche in which immune responses are profoundly shaped by local interactions between infiltrating leukocytes, epithelial cells, stromal components, and tissue-derived inflammatory signals [6]. In this setting, $CD8^+$ T lymphocytes play a central role in the control of HSV-2 infection through cytotoxic activity and the production of antiviral cytokines [7, 8]. These cells recognize infected targets through peptide presentation by MHC class I molecules and contribute not only to the elimination of infected epithelial cells but also to the suppression of viral reactivation in sensory ganglia and peripheral tissues [9]. Initial priming of these cells occurs in draining lymph nodes, followed by migration to infected tissues where local cues further shape their differentiation and functional specialization [10]. The activation and differentiation of these cells are strongly dependent on the strength of T cell receptor (TCR) signalling, which is directly influenced by antigen load and the inflammatory context present in the tissue during the acute phase of infection [11].

Among memory T-cell subsets present in barrier tissues, tissue-resident memory T cells (TRM) have emerged as key mediators of local antiviral immunity due to their capacity to permanently reside within non-lymphoid tissues and rapidly respond upon pathogen re-encounter [12, 13, 14]. In HSV-2 infection, $CD8^+$ TRM cells positioned within the genital mucosa are strategically localized at sites of previous viral replication, enabling rapid containment of recurrent viral

reactivation. However, acquisition of the resident phenotype is not a terminal event that occurs after tissue entry, but rather a progressive differentiation program initiated during the effector phase and continuously reinforced by signals derived from the infected tissue microenvironment [15, 16]. Importantly, increasing evidence indicates that antigen availability during the acute phase of infection plays a pivotal role in shaping the generation of CD8 TRM precursors [17, 18]. The magnitude and duration of TCR signaling directly influence the transcriptional and metabolic programs associated with tissue residency, including the induction of molecules involved in tissue retention and long-term persistence [19, 20]. Sustained local antigen exposure has been associated with enhanced expression of residency-associated markers such as CD69, CD103, and CD49a, as well as with transcriptional adaptations involving Runx3 and the progressive reduction of T-bet and Eomes expression [21, 22]. In parallel, tissue-derived cytokines such as TGF-β and IL-15 contribute to the stabilization and survival of developing TRM populations [18, 23, 24]. Together, these findings suggest that early antigenic and inflammatory signals critically determine the establishment, localization, and quality of the resident memory compartment.

Consistent with this concept, CD8 TRM distribution within infected tissues is frequently restricted to regions previously exposed to viral antigen, indicating that local antigen persistence contributes to the spatial organization of resident immunity [25]. This spatial organization suggests that the magnitude and localization of the immune response are largely determined by the early dynamics of infection, including viral load and antigen availability [26]. This distribution pattern enables rapid local immune responses upon viral reactivation, facilitating early containment of viral replication at previously infected sites [27]. Consequently, factors capable of modulating viral replication and antigen availability during the acute phase of infection may substantially influence the differentiation dynamics of CD8 TRM precursors and the establishment of protective mucosal immunity.

In the clinical setting, HSV-2 replication is primarily controlled through nucleoside analogues such as acyclovir and its derivatives, which remain the cornerstone of antiviral therapy. In particular, acyclovir is the standard treatment for primary HSV-2 infection, where it effectively suppresses viral replication,

reduces symptom severity, and accelerates lesion healing [28, 29]. This drug is used both for the treatment of acute episodes and as prophylaxis in individuals at higher risk of viral reactivation, such as immunocompromised patients [30]. However, because antiviral therapy profoundly reduces viral burden within infected tissues, it may also alter the antigenic landscape required for optimal $CD8^+$ T-cell activation and differentiation. In this context, reduced antigen availability may limit the strength and duration of TCR signalling, as well as exposure to inflammatory mediators that support tissue residency programming. [20, 31].

In this context, although acyclovir represents a key tool in the clinical management of HSV-2 infection, its impact on early events of T cell activation and the formation of TRMs precursors within infected tissues remains incompletely understood. Early modulation of viral load may directly affect the formation of tissue-resident populations by altering local antigen persistence during a critical window of immune programming. Therefore, understanding how viral load and antiviral therapy influence the early dynamics of $CD8^+$ T-cell activation within the genital mucosa is essential for elucidating the mechanisms controlling TRM precursor differentiation during HSV-2 infection. In this study, we sought to determine how acyclovir-mediated control of HSV-2 replication during the acute phase of infection influences the development of antiviral $CD8^+$ T-cell responses in the genital mucosa. Specifically, we investigated whether a reduced viral burden affects early $CD8^+$ T-cell differentiation and the expression of the tissue residency-associated markers CD69, CD49a, and CD103, which are linked to the generation of TRM precursor [27].

## Materials And Methods

### Virus

Herpes simplex virus 2 (HSV-2), strain 333, was expanded and titrated in Vero E6 cells (ATCC CRL-1586). Cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (LGC Biotecnologia) at 37°C in a humidified atmosphere containing 5% $CO_2$. For viral production, confluent cells were infected with HSV-2 in medium containing 2% FBS and incubated until approximately 80% cytopathic effect was observed. The supernatant containing viral particles was collected, aliquoted, and stored at −80°C. Viral titers were determined by plaque assay and expressed as PFU/mL.

### Animals and genital infection model

Female C57BL/6 mice, aged 7–10 weeks, were used in the experiments. To synchronize the estrous cycle and increase susceptibility to genital infection, animals received medroxyprogesterone via intraperitoneal injection 4–5 days prior to infection. Subsequently, mice were anesthetized via intraperitoneal administration of ketamine (100 mg/kg) and xylazine (10 mg/kg). The genital mucosa was then gently scarified with a sterile needle prior to vaginal HSV-2 inoculation. The clinical progression of infection was monitored daily using a previously established genital lesion scoring system, in a scale of 1 to 4, with each point established for erythema, hair loss, exudate, and necrosis. Animals were euthanized using three times the anesthetic dose.

### Experimental design

The study was conducted in two independent phases. In the first phase, mice were infected intravaginally with increasing doses of HSV-2 ($7.5\times10^3$, $1.5\times10^4$, and $3\times10^4$ PFU/mL) to determine an inoculum capable of inducing detectable $CD8^+$ T-cell responses in the genital mucosa and to evaluate the influence of viral load on these responses. Based on the results obtained in this initial dose-titration experiment, a single viral dose was selected for the second

phase of the study. In this phase, animals were allocated into experimental groups with or without acyclovir treatment. Antiviral therapy was administered orally through the drinking water at a concentration of 1.5 mg/mL starting after infection, and an additional intraperitoneal dose of acyclovir (40 mg/kg) was administered on day 5 post-infection [32, 33].

Animals were euthanized at the end of the acute phase of infection (14 days post-infection) for the collection of draining lymph nodes, spleen, and genital tissue samples. All experiments were performed using independent groups of animals and were repeated to ensure the reproducibility of the results.

## Tissue Processing

Genital tissue was subjected to enzymatic digestion with collagenase IV (Thermo-Fischer) (3 mg/mL) and DNase (Thermo-Fischer) (0.2 mg/mL) in RPMI (Sigma-Aldrich) culture medium in water bath for 45min at 37°C to release cells from the tissue, followed by filtration and mechanical dissociation through a cell strainer to obtain a single-cell suspension [34]. Lymph nodes were mechanically dissociated and filtered through a cell strainer to obtain single-cell suspensions. Spleen samples were processed in the same manner and subsequently subjected to red blood cell lysis to remove erythrocytes and enrich the leukocyte fraction. During genital tissue processing, the supernatant was collected and stored for subsequent cytokine analysis.

## Flow Cytometry

Single-cell suspensions obtained from the genital tissue, draining lymph nodes, and spleen were used for phenotypic characterization of T-cell populations by flow cytometry. Prior to antibody staining, Fc receptors were blocked using FcBlock (BioLegend) to minimize nonspecific antibody binding. Cells were then stained with a viability dye to exclude dead cells, followed by incubation with fluorochrome-conjugated monoclonal antibodies directed against the surface markers CD3, CD4, CD8, CD44, CD45, CD69, CD103, CD49a, and PD-1 (BD Biosciences). These markers were used to identify activated T cells and characterize TRM precursor and resident populations.

To determine the absolute number of immune cell subsets, counting beads (BD Biosciences) were added to each sample according to the manufacturer's instructions. After staining, samples were acquired on a BD FACSymphony™ A5 flow cytometer (BD Biosciences), and compensation was performed using single-stained controls. Data were analyzed using FlowJo software (Tree Star Inc.), where sequential gating strategies were applied to exclude debris, doublets, and nonviable cells, followed by the identification and quantification of the T-cell populations of interest based on the expression of specific phenotypic markers.

## Cytokine quantification

Cytokine levels of TNF-α, IL-21 and IFN-γ in genital tissue supernatants were determined using a Cytometric Bead Array (CBA) (BD Biosciences) assay with commercial kits, according to the manufacturer's instructions. Samples were analyzed on a BD FACSCanto™ II flow cytometer.

## Statistical analysis

Data were expressed as mean ± standard deviation (SD). For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used. For experiments involving only two groups, an unpaired Student's t-test was applied. Analyses were performed using GraphPad Prism software, and p values $< 0.05$ were considered statistically significant.

## Results

### HSV-2 Inoculum Dose Determines Disease Severity, Weight Loss, and Mortality Following Intravaginal Infection

To investigate the impact of viral burden on disease progression and the subsequent immune response, C57BL/6 mice were intravaginally infected with increasing doses of HSV-2 (Low: $7.5\times10^3$, Intermediate: $1.5\times10^4$, or High: $3\times10^4$ PFU/mL) (Fig. 1A). Disease progression was monitored using a standardized genital lesion scoring system (Fig. 1B-C). Mice infected with higher viral doses developed more severe genital lesions, experienced greater body weight loss, and exhibited increased mortality compared with animals receiving lower inocula, which displayed milder clinical manifestations and better preservation of body weight throughout the infection course (Fig. 2A-B). These findings indicate a direct relationship between the initial viral inoculum and disease severity.

To further characterize disease kinetics, clinical scores were analysed over the course of infection. Mice infected with the highest viral dose developed severe disease more rapidly and succumbed earlier than animals in the other groups (Fig. 2C). Consequently, the average clinical score in this group declined at later time points due to the loss of the most severely affected animals. In contrast, mice infected with intermediate and low viral doses exhibited delayed disease progression and mortality, resulting in a more prolonged persistence of genital lesions and sustained clinical scores throughout the observation period (Fig. 2D). A similar pattern was observed for body weight variation, with the highest-dose group showing earlier and more pronounced weight loss, whereas lower-dose groups displayed a more gradual decline and improved recovery over time (Fig. 2B).

Collectively, these results demonstrate that the initial HSV-2 inoculum is a critical determinant of disease outcome. Higher viral loads drive more severe clinical manifestations, accelerated weight loss, and earlier mortality, establishing a robust dose-dependent model for investigating how viral burden shapes antiviral immune responses during genital HSV-2 infection. Importantly, a detailed understanding of disease kinetics across different inoculum doses is essential for the rational design of immunological memory studies, as it enables

the selection of experimental conditions that ensure animal survival while allowing the development and long-term maintenance of memory T-cell responses following primary infection.

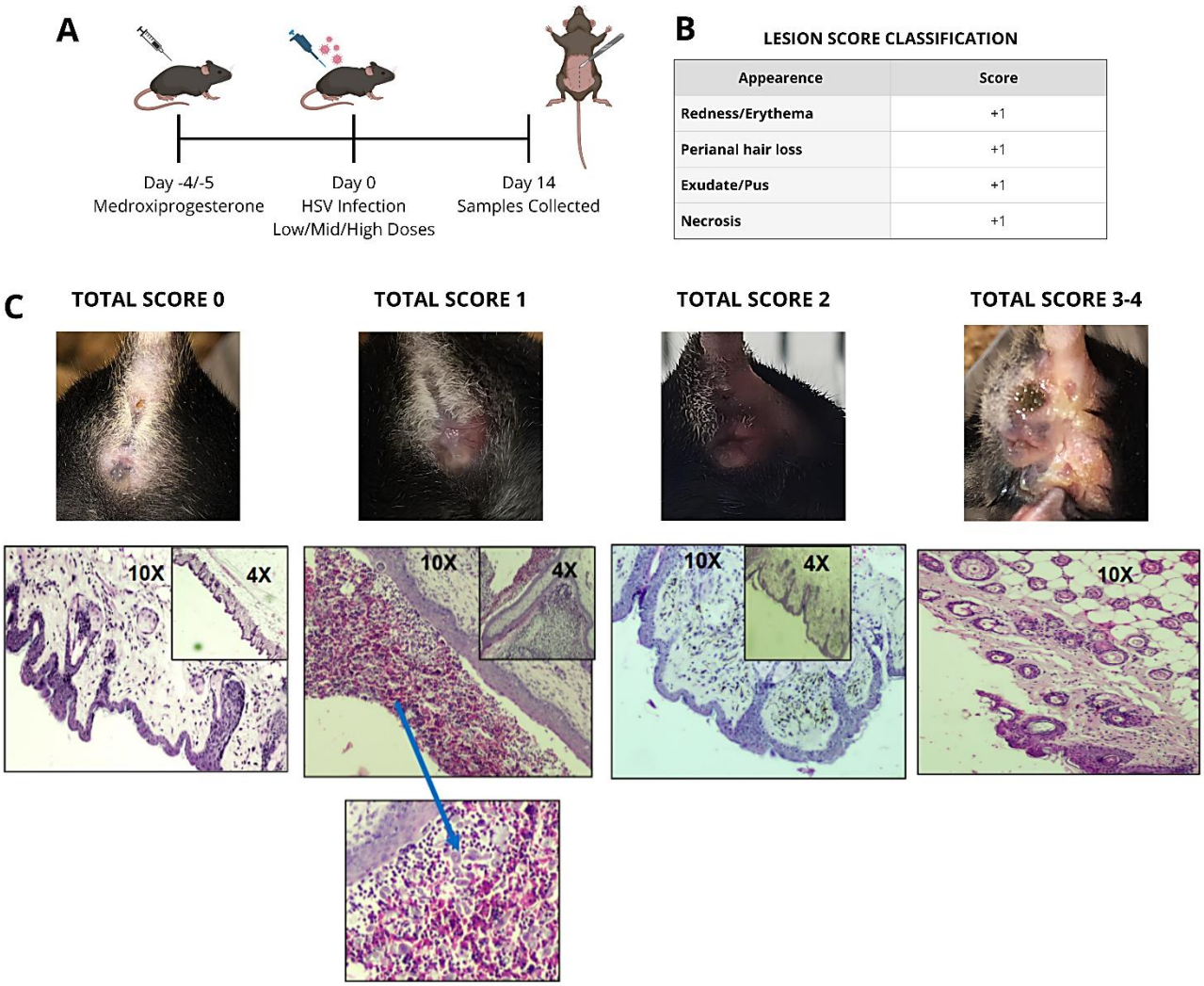



**Figure 1 - Clinical and pathological parameters of genital HSV-2 infection** (A) Experimental design: C57BL-6 mice treated with medroxyprogesterone were infected with Low - $7.5\times10^3$, Mid - $1.5\times10^4$, or High - $3\times10^4$ PFU/mL of HSV-2. Fourteen days after infection the animal were euthanised and the immunological analysis performed. (B-C) Classification of genital lesion scoring in mice with HE-stained histological images.

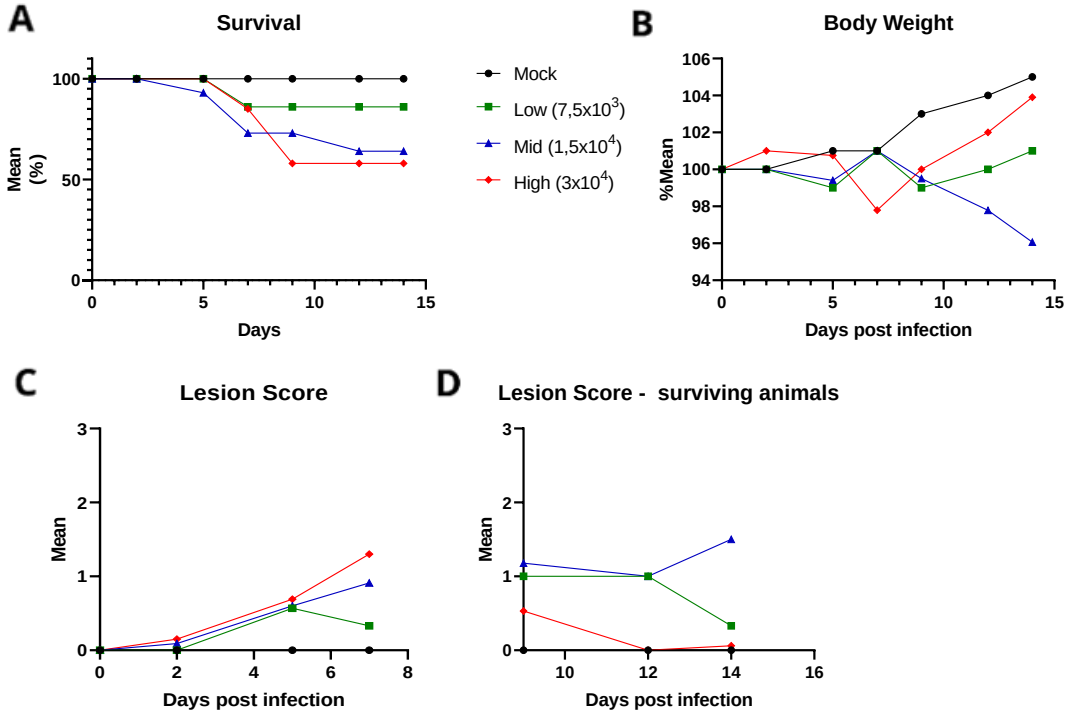



**Figure 2 - Clinical scores of genital HSV-2 infection** (A) Survival of animals over 14 days of infection with different doses; (B) Mean body weight represented as percentage of weight gain relative to initial weight; (C-D) Mean lesion score up to day 7 of infection and from day 7 onward, respectively.

## Increasing HSV-2 Inoculum Promotes Local Expansion of CD44⁺ Effector CD8⁺ T Cells

Given that increasing HSV-2 inoculum doses resulted in greater disease severity, weight loss, and mortality, we next investigated whether these differences were associated with distinct local $CD8^+$ T-cell responses within the genital mucosa. At 14 days post-infection, genital tissues were collected, enzymatically processed, and analyzed by flow cytometry (Supl. Fig. 1). Compared with noninfected controls, HSV-2-infected mice exhibited a marked increase in the frequency and absolute number of $CD3^+CD8^+$ T lymphocytes in the genital tissue, demonstrating that genital HSV-2 infection promotes the recruitment and/or expansion of local $CD8^+$ T cells (Fig. 3A-B). Phenotypic characterization further revealed that most of these cells displayed an activated/effector phenotype, characterized by CD44 expression ($CD3^+CD8^+CD44^+$). Importantly, the accumulation of $CD44^+$ effector $CD8^+$ T cells

occurred in a dose-dependent manner, with progressively higher frequencies observed in mice infected with increasing viral inocula (Fig. 3C). Animals receiving the highest HSV-2 dose exhibited the most robust expansion of genital $CD3^+CD8^+CD44^+$ T cells, indicating that greater antigenic exposure drives a stronger local effector T-cell response.

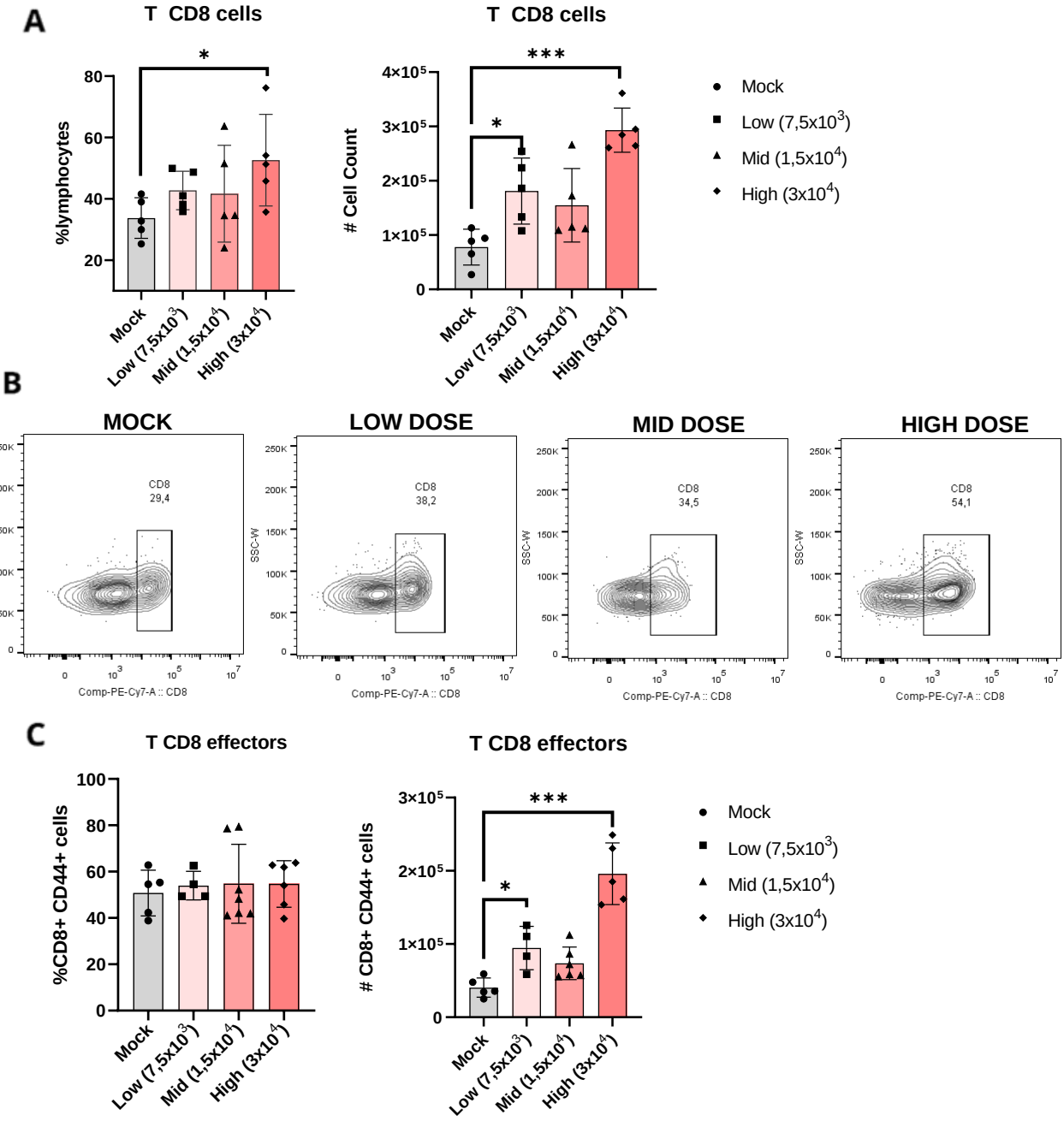



**Figure 3 - Initial HSV-2 inoculum modulates genital $CD8^+$ T-cell accumulation and activation:** (A-B) Percentage and absolute count, respectively, of $CD8^+$ T lymphocytes in genital tissue; (C) Percentage and

absolute count, respectively, of $CD8^+$ $CD44^+$ T lymphocytes in genital tissue. (*) p value < 0.05; (***) p value < 0.01.

These findings establish that the same viral doses associated with increased clinical disease severity also promote enhanced accumulation of effector $CD8^+$ T lymphocytes within the genital mucosa. Thus, the initial viral burden not only determines the clinical outcome of HSV-2 infection but also directly shapes the magnitude of the local cellular immune response. Because $CD44^+$ effector $CD8^+$ T cells constitute the precursor population from which tissue-resident memory T cells are generated, these results further suggest that viral inoculum may influence the subsequent establishment of antiviral immune memory in the genital tract.

**A Threshold of Viral Burden Is Required for the Acquisition of Tissue-Resident Memory-Associated Phenotypes by Genital $CD8^+$ T Cells**

Given the dose-dependent expansion of genital effector $CD8^+$ T cells observed following HSV-2 infection, we next investigated whether the magnitude of the initial viral inoculum influenced the generation of cells expressing tissue-residency-associated markers after the acute phase of infection. To this end, the expression of the canonical residency markers CD69, CD103, and CD49a was evaluated within genital $CD3^+CD8^+CD44^+$ effector T cells. Four populations associated with progressive stages of tissue-resident memory T-cell (TRM) differentiation were characterized: $CD69^+$, $CD69^+CD103^+$, $CD69^+CD49a^+$ and $CD69^+CD103^+CD49a^+$ cells (Fig. 4A-D). HSV-2 infection promoted the accumulation of $CD8^+$ T cells expressing residency-associated markers within the genital mucosa when compared with noninfected controls. However, a marked increase in these populations was observed primarily in mice infected with the highest viral dose ($3 \times 10^4$ PFU/mL). Animals receiving this inoculum exhibited significantly greater numbers of $CD69^+$, $CD69^+CD103^+$, $CD69^+CD49a^+$ and $CD69^+CD103^+CD49a^+$ $CD8^+$ T cells, whereas lower viral doses induced only modest or nonsignificant changes in these populations (Fig. 4A-D). These findings indicate that the establishment of a tissue-resident differentiation

program requires a strong local antigenic stimulus and is favored under conditions of elevated viral burden.

Further phenotypic analysis revealed a significant enrichment of CD49a-expressing cells among the residency-associated populations generated in the high-dose group (Fig. 4C-E). Because CD49a has been associated with enhanced tissue retention, cytotoxic function, and long-term persistence of mucosal TRM cells, these data suggest that higher viral loads not only increase the number of TRM precursors but also promote their progression toward a more mature resident phenotype.

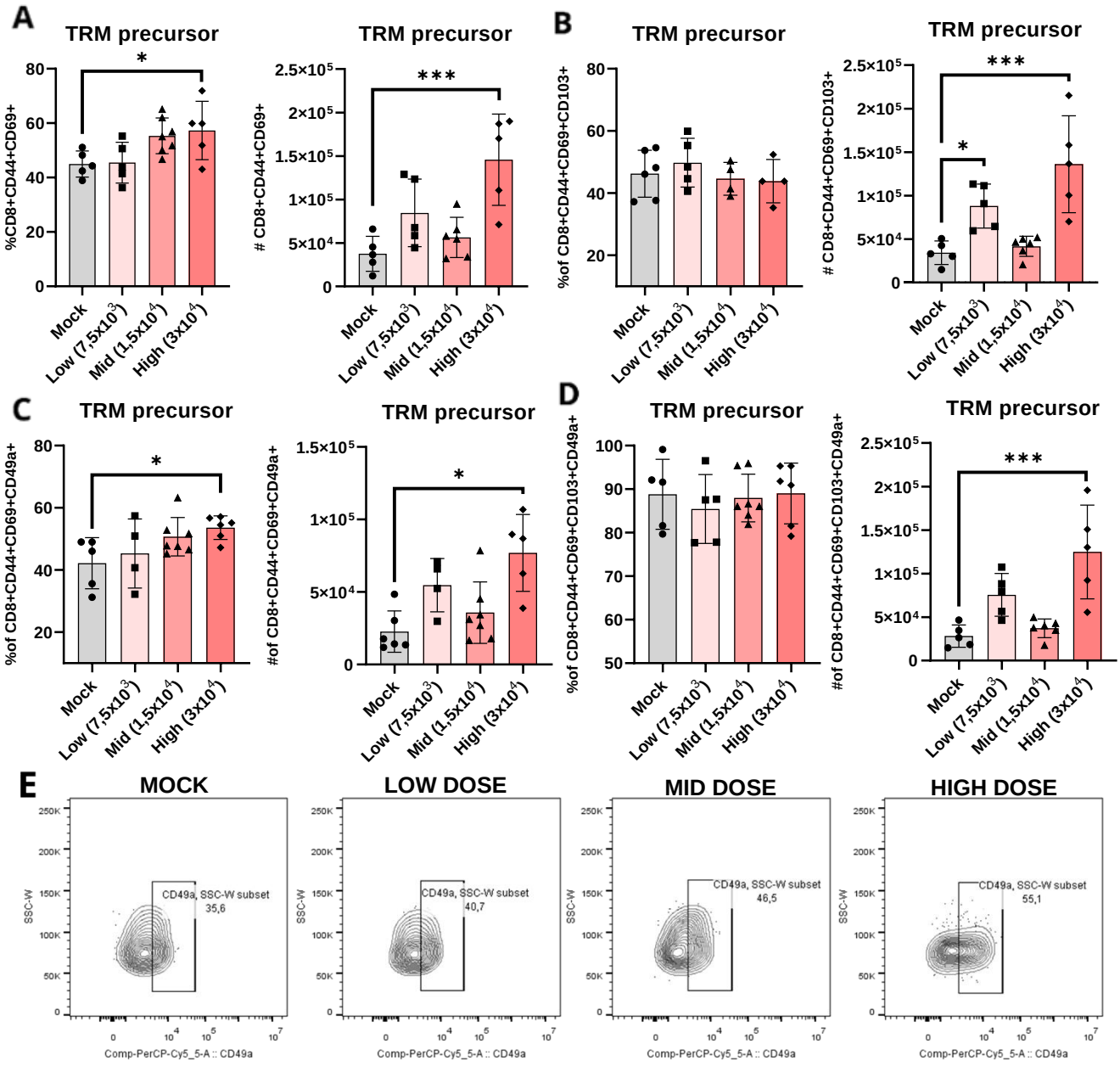


**Figure 4 - Local viral burden preferentially drives the local generation of CD8⁺ TRM precursors in genital mucosa:** (A) Percentage and absolute count of CD69⁺ CD103⁻ lymphocytes in genital tissue; (B) Percentage and absolute count of CD69⁺ CD103⁺ lymphocytes in genital tissue; (C) Percentage and absolute count of CD69⁺ CD49a⁺ lymphocytes in genital tissue; (D) Percentage and absolute count of CD69⁺ CD103⁺ CD49a⁺ lymphocytes in genital tissue; (E) Different HSV doses and CD49a⁺ lymphocyte population in genital tissue; (*) p value < 0.05; (***) p value < 0.01.

To investigate whether these changes were restricted to the site of infection or reflected broader systemic immune modulation, splenic CD8⁺ T-cell populations were analyzed following mechanical dissociation and erythrocyte lysis. In contrast to the pronounced alterations observed in genital tissue, most splenic CD8⁺ T-cell and TRM precursor populations (CD8⁺CD69⁺CD103⁺CD49a⁺) did not differ significantly among experimental groups (Fig. 5A–F). Nevertheless, infected animals exhibited increased expression of the activation marker CD44 (Fig. 5B), indicating the presence of systemic immune activation despite the limited expansion of circulating TRM-related populations.

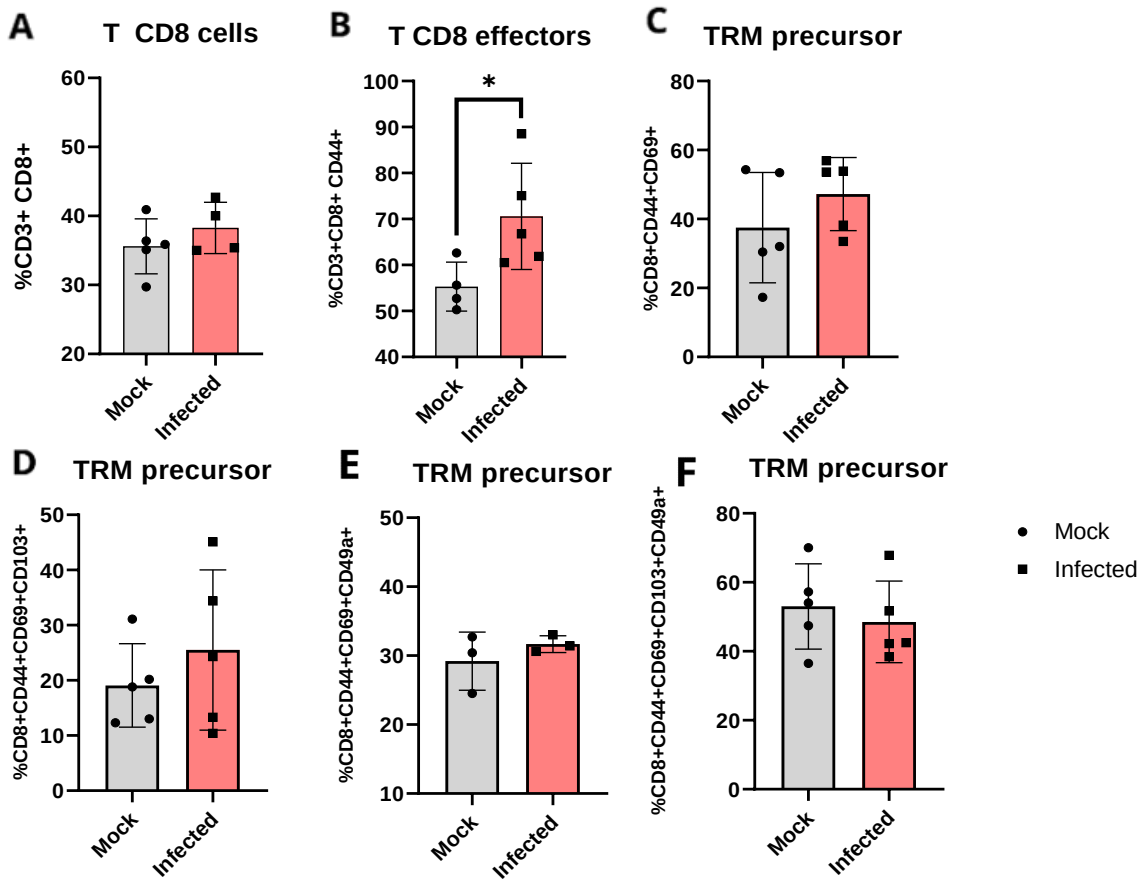


**Figure 5 - Differences in percentage between infected groups and the non-infected group of lymphocytes in the spleen.:** (A) $CD8^+$ T cells; (B) $CD44^+$; (C) $CD69^+$ $CD103^-$ (D) $CD69^+$ $CD103^+$; (E) $CD69^+$ $CD49a^+$; (F) $CD69^+$ $CD103^+$ $CD49a^+$. (*) p value < 0.05.

Collectively, these results demonstrate that the initial HSV-2 inoculum critically influences the generation of $CD8^+$ T cells expressing tissue-residency markers within the genital mucosa. While all infection doses induced the expansion of effector $CD8^+$ T cells, robust acquisition of TRM-associated phenotypes was observed predominantly in mice infected with $3\times10^4$ PFU/mL, suggesting that a threshold level of antigenic exposure is required to efficiently drive the local differentiation of resident memory T-cell precursors. These findings identify the highest viral inoculum as the condition most favorable for the establishment of genital $CD8^+$ T-cell populations with resident-memory characteristics.

## Acyclovir-Mediated Suppression of HSV-2 Replication Impairs the Generation of Genital $CD8^+$ T Cells with Tissue-Resident Memory Characteristics

Having demonstrated that higher HSV-2 inoculum promotes the accumulation of effector $CD8^+$ T cells and favours the acquisition of tissue-residency-associated phenotypes within the genital mucosa, we next investigated whether reducing viral replication during primary infection would impact the generation of these populations. To address this question, HSV-2-infected mice were treated or not with acyclovir, and genital $CD8^+$ T-cell responses were evaluated following infection (Fig. 6A).

The efficacy of acyclovir treatment was supported by improved clinical parameters, including a trend toward greater average weight gain, lower genital lesion scores, and reduced mortality in treated animals (Fig. 6B–D).

Acyclovir treatment resulted in a significant reduction in both the frequency and absolute number of genital $CD3^+CD8^+$ T lymphocytes when compared with untreated infected animals (Fig. 6E). Consistent with this finding, the population of effector $CD8^+$ T cells ($CD3^+CD8^+CD44^{high}$) was also markedly reduced following antiviral treatment (Fig. 6F), indicating that suppression of viral

replication limits the local expansion and activation of virus-responsive T cells. The CD44$^{high}$ gate was applied to selectively identify highly activated CD8$^{+}$ T cells and exclude cells expressing basal levels of CD44.

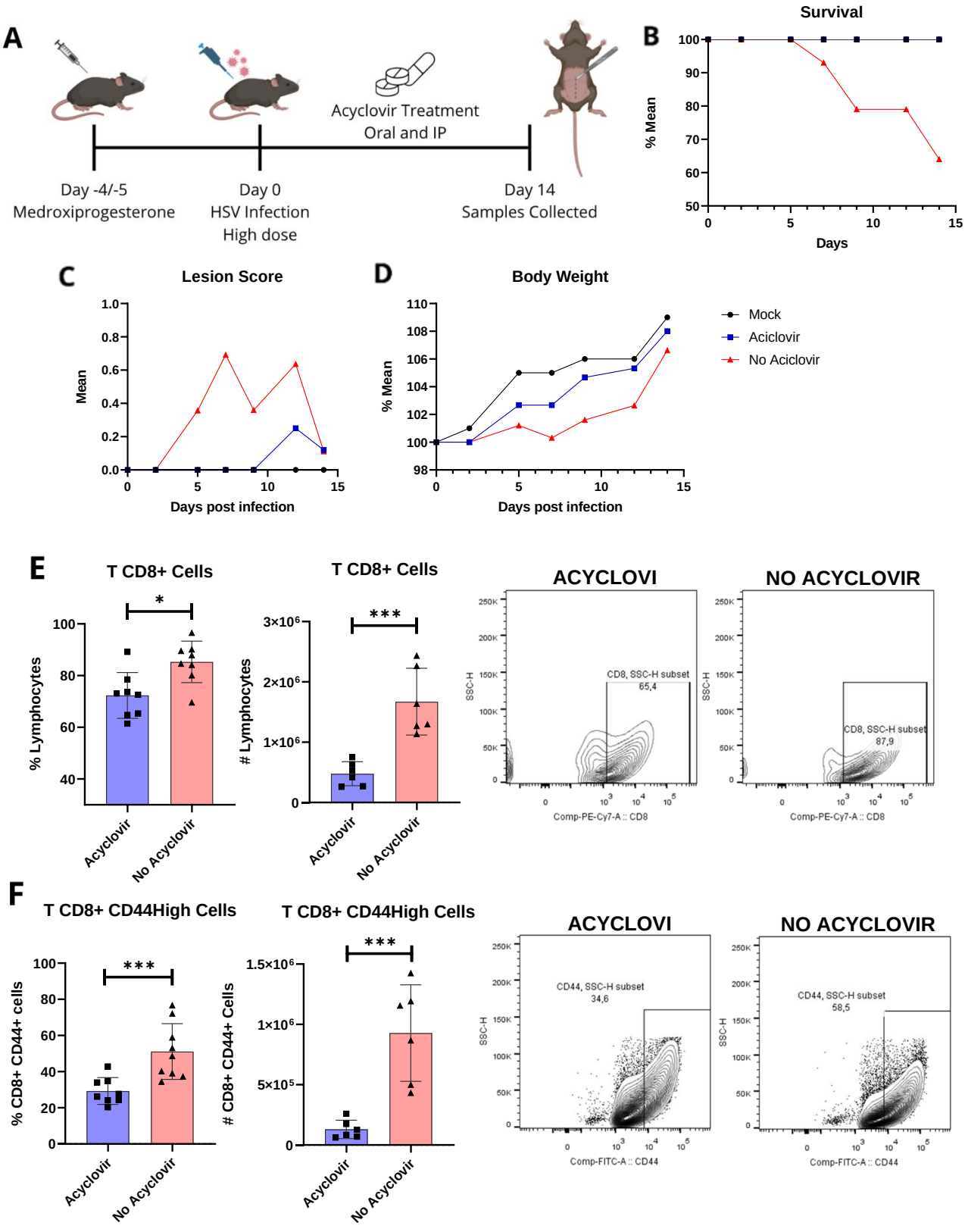


**Figure 6 - Acyclovir treatment modulates the acute local T CD8$^+$ immune response in the genital tissue:** (A) Experimental design with antiviral treatment using acyclovir; (B) Survival; (C) Lesion score; (D) Mean body weight; (E) Percentage and absolute count of T CD8$^+$ lymphocytes in genital tissue; (F) Percentage and absolute count of activated T CD8$^+$ lymphocytes (CD44$^{high}$) in genital tissue; (*) p value < 0.05; (***) p value < 0.01.

To determine whether reduced antigenic exposure also affected the establishment of tissue-residency-associated populations, we evaluated the expression of canonical TRM markers within genital CD8$^+$ T cells. Interestingly, no significant differences were observed in the frequency of CD3$^+$CD8$^+$CD69$^+$ cells between untreated and acyclovir-treated animals (Fig. 7A), suggesting that expression of CD69 alone is not sufficient to reflect the impact of viral replication on the tissue-residency program. In contrast, when additional residency markers were incorporated into the analysis, a distinct effect of antiviral treatment became evident. Acyclovir administration led to an approximately threefold reduction in the frequency and number of both CD3$^+$CD8$^+$CD69$^+$CD103$^+$ and CD3$^+$CD8$^+$CD69$^+$CD103$^+$CD49a$^+$ populations (Fig. 7B-C), indicating a profound impairment in the generation of CD8$^+$ T cells displaying a mature tissue-resident phenotype.

Because persistent antigen exposure during HSV infection has been associated with the induction of PD-1 expression on antigen-experienced CD8$^+$ T cells [35], we further evaluated this marker as an additional indicator of local T-cell differentiation. In agreement with the reduction observed in TRM-associated populations, acyclovir-treated animals exhibited a significant decrease in the frequency of PD-1-expressing CD8$^+$ T cells within the genital mucosa (Fig. 7D). This finding further supports the notion that antiviral treatment alters the local antigenic environment and consequently limits the differentiation of activated CD8$^+$ T cells.

Taken together, these findings demonstrate that viral replication is a critical driver of the tissue-residency program induced during genital HSV-2 infection. Antiviral suppression of viral replication significantly reduced the accumulation of effector CD8$^+$ T cells and markedly impaired the generation of CD69$^+$CD103$^+$ and CD69$^+$CD103$^+$CD49a$^+$ populations within the genital mucosa. These data indicate that sustained local antigenic stimulation is required not only for the

expansion of antiviral CD8⁺ T cells but also for their differentiation toward tissue-resident memory precursor populations. Thus, the magnitude and duration of antigen exposure emerge as key factors governing the establishment of local CD8⁺ T-cell memory during genital HSV-2 infection.

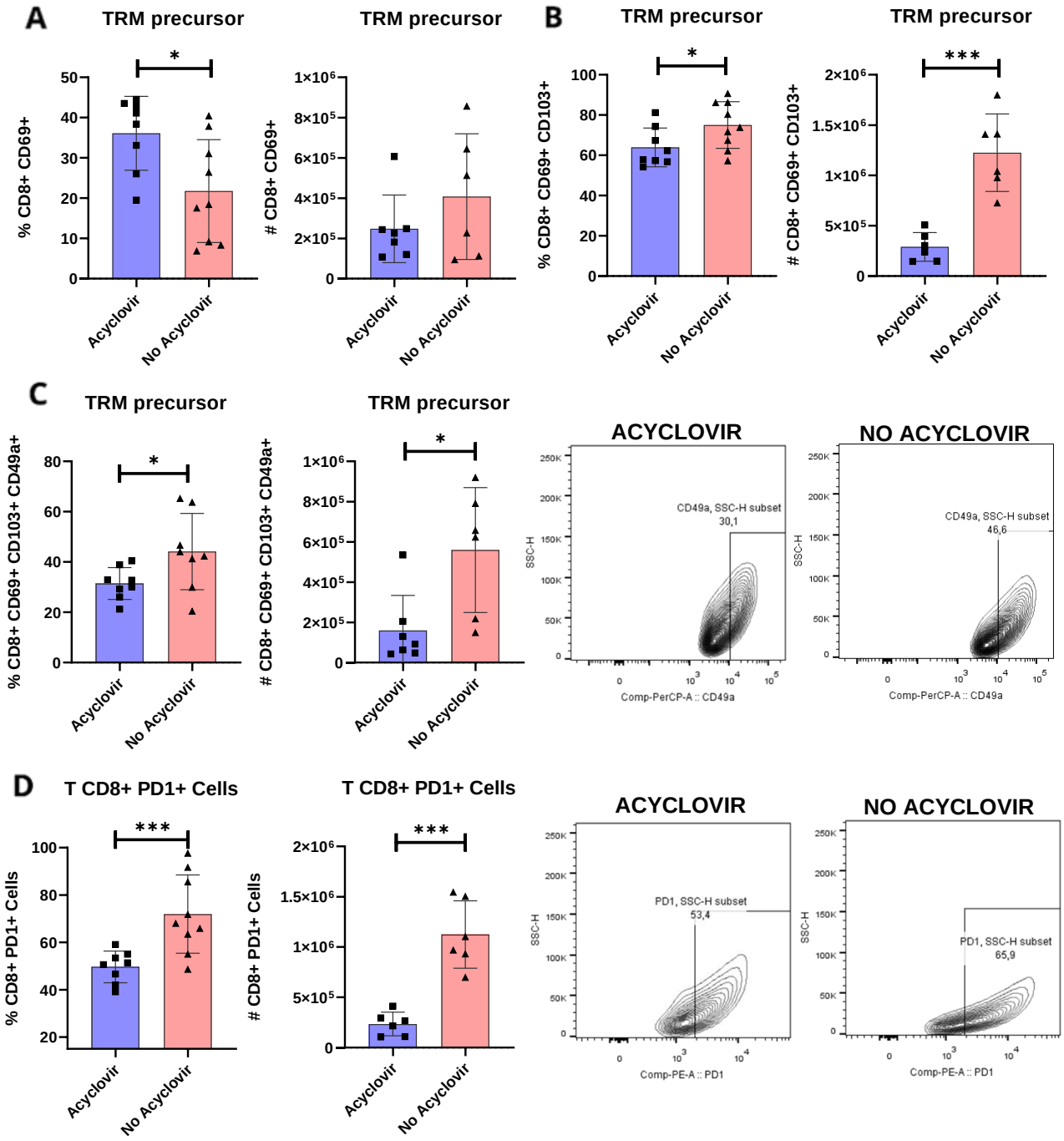



**Figure 7 - Acyclovir treatment modulates the acute local TRM precursors immune response in the genital tissue:** (A) Percentage and absolute count of CD8⁺CD69⁺ lymphocytes in genital tissue; (B) Percentage and

absolute count of $CD8^+CD69^+CD103^+$ lymphocytes in genital tissue; (C) Percentage and absolute count of $CD8^+CD69^+CD103^+CD49a^+$ lymphocytes in genital tissue; (D) Percentage and absolute count of $CD8^+PD1^+$ lymphocytes in genital tissue; (*) p value < 0.05; (***) p value < 0.01.

**TRM-Associated $CD8^+$ T Cell Populations Are Preferentially Generated in the Genital Mucosa and Not in Secondary Lymphoid Organs**

To determine whether the effects of viral replication on $CD8^+$ T-cell differentiation extended beyond the site of infection, we next evaluated $CD8^+$ T-cell populations in the inguinal draining lymph nodes and spleen following acyclovir treatment. In contrast to the pronounced changes observed in the genital mucosa, antiviral treatment had only a limited impact on $CD8^+$ T-cell populations within secondary lymphoid organs. HSV-2 infection resulted in an increase in the total number of $CD8^+$ T cells in both the inguinal lymph nodes and spleen compared with noninfected controls (Fig. 8A and 9A). However, despite this expansion, no significant differences were detected among experimental groups in the frequencies or numbers of effector $CD8^+$ T cells ($CD8^+CD44^{high}$) or in populations expressing tissue-residency-associated markers, including $CD8^+CD44^+PD1^+$, $CD8^+CD44^+CD69^+CD103^+$, and $CD8^+CD44^+CD69^+CD103^+CD49a^+$ cells (Fig. 8-9).

Moreover, acyclovir treatment did not significantly alter the distribution of these populations in either the draining lymph nodes or the spleen, contrasting sharply with the marked reduction in effector and TRM precursor populations observed within the genital tissue. These findings indicate that the impact of viral replication on the acquisition of tissue-residency-associated phenotypes is largely restricted to the local site of infection.

# SPLEEN

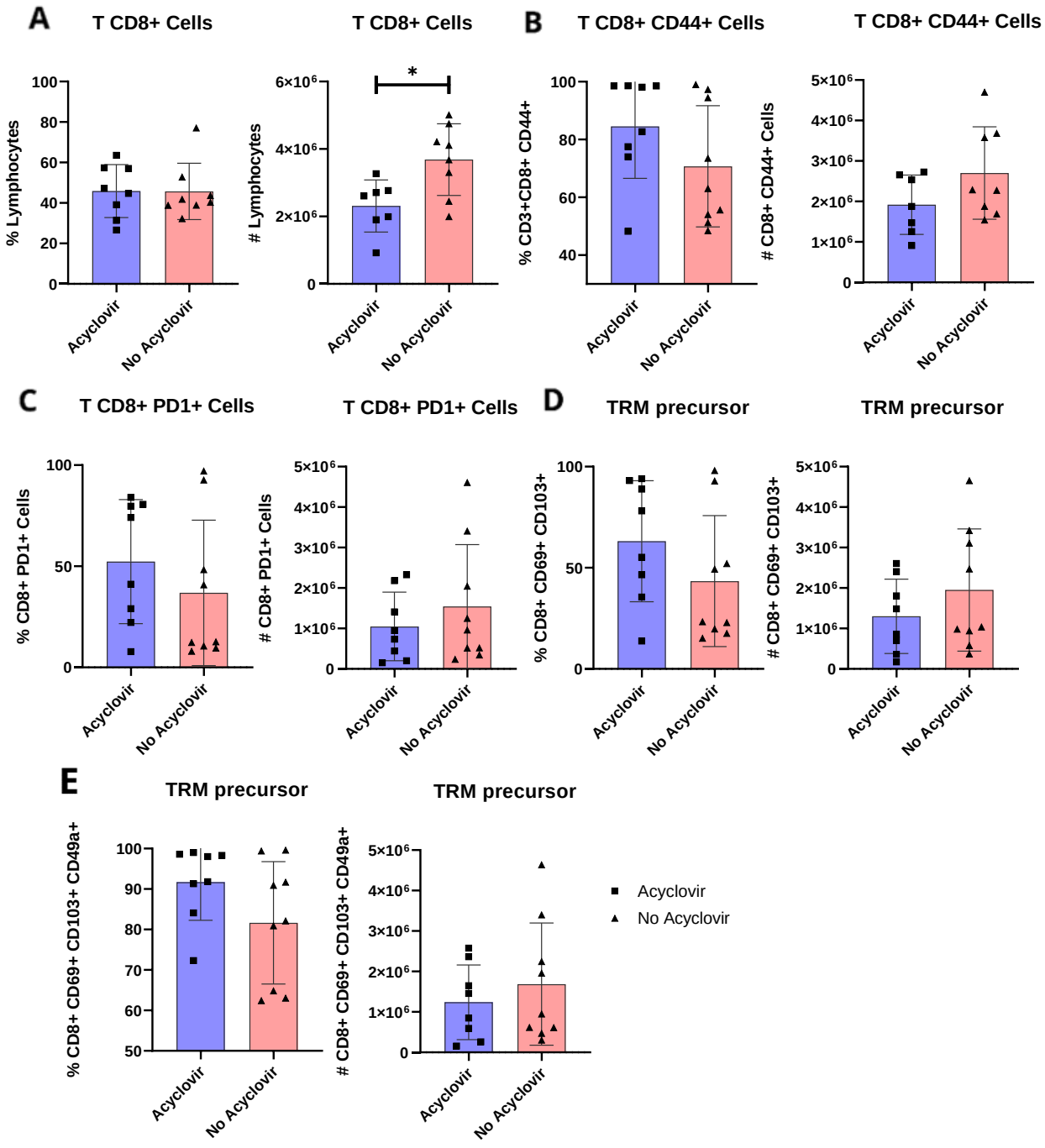



**Figure 8 - The impact of acyclovir on the systemic immune response is limited in Spleen:** (A) Percentage and absolute count of T CD8$^+$ lymphocytes in the spleen; (B) Percentage and absolute count of T CD8$^+$CD44$^+$ lymphocytes; (C) Percentage and absolute count of CD8$^+$CD44$^+$PD1$^+$ cells; (D) Percentage and absolute count of CD8$^+$CD44$^+$CD69$^+$CD103$^+$ cells; (E) Percentage and absolute count of CD8$^+$CD44$^+$CD69$^+$CD103$^+$CD49a$^+$ cells; (*) p value < 0.05.

# LYMPH NODE

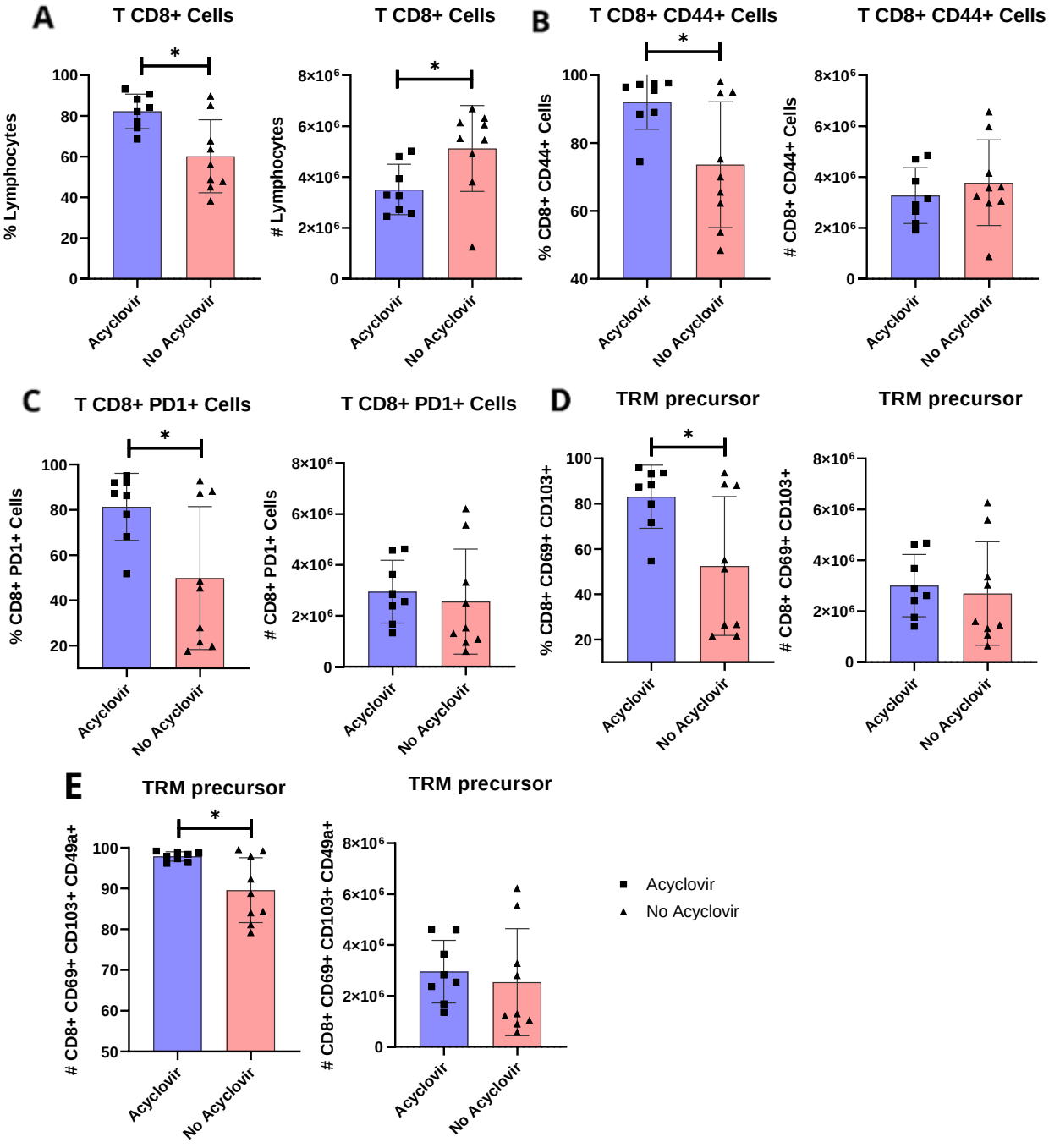



**Figure 9 - The impact of acyclovir on the systemic immune response is limited in draining Lymph node:** (A) Percentage and absolute count of T $CD8^+$ lymphocytes in the draining lymph node; (B) Percentage and absolute count of T $CD8^+CD44^+$ lymphocytes; (C) Percentage and absolute count of $CD8^+CD44^+PD1^+$ cells; (D) Percentage and absolute count of $CD8^+CD44^+CD69^+CD103^+$ cells; (E) Percentage and absolute count of $CD8^+CD44^+CD69^+CD103^+CD49a^+$ cells; (*) p value < 0.05.

In addition, to assess the impact of antiviral treatment on the local inflammatory response, cytokine levels were measured in genital tissue supernatants using a Cytometric Bead Array (CBA) assay. Cytokines associated with antiviral responses and T cell activation, including TNF, IL-21 and IFN-γ were analyzed (Fig. 10A–C). Comparison between treated and untreated groups did not reveal a consistent pattern in cytokine levels within the genital tissue, with variations observed across groups that did not follow a clear trend. These results indicate that, despite the cellular changes observed, antiviral treatment does not induce detectable or consistent alterations in the cytokine profile at the evaluated time point.

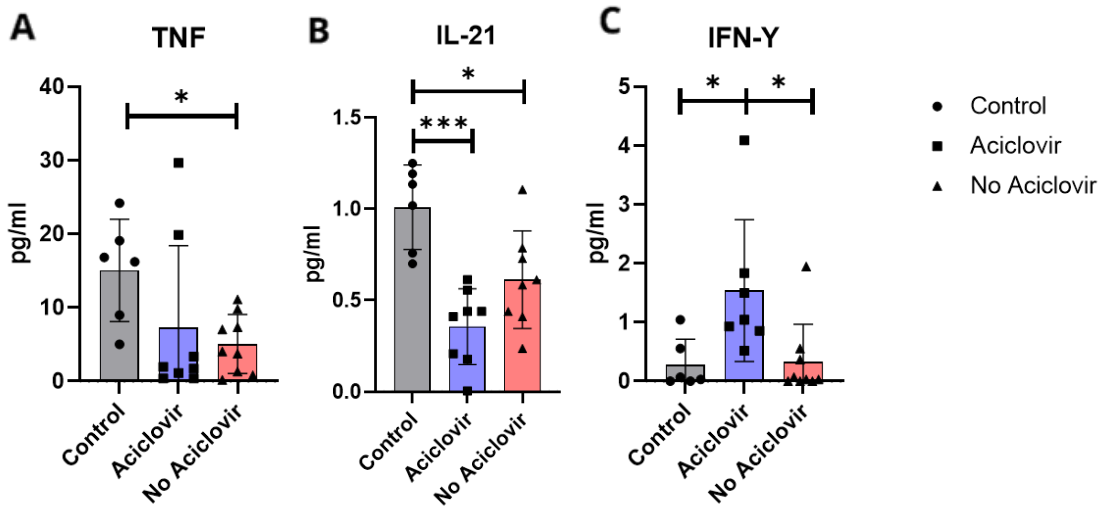



**Figure 10: Cytometric bead array (CBA) from genital tissue extract:** (A) TNF; (B) IL-21; (C) IFN-γ. (*) p value < 0.05; (***) p value < 0.01.

Together, these results suggest that the expression of CD8 TRM-associated markers during genital HSV-2 infection is driven primarily by tissue-specific cues within the infected mucosa rather than by systemic immune activation. While HSV-2 infection promotes expansion of $CD8^+$ T cells in secondary lymphoid organs, the differentiation of $CD8^+$ T cells toward a resident-memory precursor phenotype appears to occur preferentially within the genital tissue, where local antigen exposure and inflammatory signals are likely to be most prominent.

## Discussion

The establishment of effective local immunity following HSV-2 infection depends on the coordinated activation, expansion, and differentiation of virus-specific $CD8^+$ T cells within infected mucosal tissues [36, 37] . Beyond their immediate effector functions, activated $CD8^+$ T cells can acquire TRM program that enables long-term persistence at sites of previous viral replication and provides rapid protection against recurrent infection and viral reactivation [6, 20]. In the present study, we demonstrate that both the magnitude of the initial viral inoculum and acyclovir-mediated suppression of viral replication profoundly influence the early generation of $CD8^+$ T cells expressing residency-associated markers in the genital mucosa. Together, our findings support the concept that antigen availability during acute HSV-2 infection is a critical determinant of early TRM precursor formation.

Our findings indicate that the magnitude of antigenic stimulation during the priming and effector phases of HSV-2 infection critically influences the formation of $CD8^+$ T-cell responses within the genital tissue. Recent evidence suggests that TRM differentiation is initiated during the effector phase and is progressively reinforced by local antigen recognition, inflammatory mediators, and tissue-derived signals that promote the expression of residency-associated molecules such as CD69, CD103, and CD49a [38, 39]. In this context, reduced viral inoculum resulted in a diminished generation of $CD8^+$ T cells expressing residency-associated markers, suggesting that suboptimal antigen exposure fails to efficiently support the differentiation of TRM precursors. These findings are consistent with current models proposing that local antigen availability functions as a critical regulator of tissue-resident memory programming by shaping TCR-dependent signalling pathways and downstream transcriptional networks associated with tissue adaptation and retention [11]. Thus, greater antigen availability during primary HSV-2 infection appears to create conditions that favours robust local T-cell activation. Beyond the expansion of $CD44^+$ effector cells, higher viral doses preferentially promoted the accumulation of $CD69^+CD103^+$ and $CD69^+CD103^+CD49a^+$ populations, suggesting that a threshold level of antigenic stimulation is required for efficient acquisition of tissue residency-associated phenotypes. Similar observations have been reported in

multiple infection models where local antigen recognition within peripheral tissues drives the induction of CD103 and stabilization of the TRM differentiation program [40, 41]. Together, these findings support the notion that antigen load is not merely a determinant of clonal expansion but also a quantitative regulator of tissue-resident memory precursor formation in HSV-2 infection.

The importance of antigen availability became even more evident following antiviral intervention. Acyclovir treatment significantly reduced the number of total and activated $CD8^+$ T cells in the genital mucosa and markedly decreased the frequency of cells expressing combinations of residency-associated markers. Interestingly, CD69 expression alone was relatively preserved, whereas $CD103^+$ and $CD49a^+$ subsets were substantially reduced (Fig. 7A-C). These results suggest that pharmacological control of viral load can directly impacts the intensity and duration of antigenic signalling required for T-cell activation and differentiation within the infected tissue. According to current models of TRM development, sustained TCR stimulation acts in concert with tissue-derived factors such as TGF-β, IL-15, and inflammatory cytokines to induce transcriptional programs associated with tissue retention, including pathways involving Runx3, Hobit, and Blimp-1 [42, 43]. Although these molecular pathways were not directly evaluated in the present study, the marked reduction of $CD69^+CD103^+$ and $CD69^+CD103^+CD49a^+$ populations following acyclovir treatment is consistent with the hypothesis that reduced antigen availability limits the progression of effector $CD8^+$ T cells toward a resident-memory precursor state. Interestingly, acyclovir treatment had a more pronounced effect on $CD103^+$ and $CD49a^+$ subsets than on CD69 expression alone. This observation suggests that initial tissue retention signals may still occur despite reduced viral replication, whereas progression toward a more mature resident phenotype requires sustained antigenic and microenvironmental stimulation. CD69 is rapidly induced following T-cell activation and functions primarily by preventing tissue egress through inhibition of S1PR1 signalling [44]. In contrast, CD103 and CD49a are more closely associated with long-term tissue adaptation, epithelial anchoring, and functional maturation of TRM cells. Therefore, the differential modulation of these markers may reflect distinct stages of the tissue residency program that exhibit different sensitivities to antigen availability.

Consistent with this interpretation, acyclovir-treated animals also exhibited reduced frequencies of PD-1-expressing $CD8^+$ T cells in the genital tissue (Fig. 7D). Although PD-1 is frequently associated with T-cell exhaustion, its expression is rapidly induced following TCR activation and may represent recent antigen stimulation during acute immune responses rather than dysfunctional differentiation, as previously observed in HSV infection model [45, 46]. Therefore, the reduction in PD-1 expression observed in treated animals likely reflects diminished antigenic stimulation resulting from reduced viral replication rather than impaired cellular function. The reduction in PD-1 expression observed after acyclovir treatment likely reflects diminished antigen encounter during the acute phase rather than functional exhaustion. The absence of major changes in secondary lymphoid organs despite substantial alterations in genital tissue supports the notion that TRM precursor differentiation is predominantly regulated by tissue-specific signals rather than systemic immune activation. These results highlight the importance of tissue-specific signals in shaping TRM differentiation and are consistent with previous studies demonstrating that residency programs are established primarily within peripheral tissues rather than secondary lymphoid organs [39, 47]. The local microenvironment provides a unique combination of antigen presentation, inflammatory mediators, and tissue-derived factors that collectively drive the acquisition of residency-associated phenotypes. Thus, while systemic immune activation occurs following HSV-2 infection, the development of TRM precursors appears to be governed predominantly by events occurring within the infected mucosa.

The concept that local antigen exposure regulates tissue-specific memory formation is not unique to HSV-2 infection. Similar mechanisms have been described in other viral infections affecting peripheral tissues, including dengue virus infection, where activated $CD8^+$ T cells rapidly acquire residency-associated markers within the skin during the acute phase of disease [48]. These observations support the broader notion that peripheral tissues serve as active sites of immune programming, where local antigen persistence influences the differentiation and long-term maintenance of resident memory populations.

The implications of these findings are particularly relevant in the context of HSV-2 biology. Because HSV-2 establishes lifelong latency in sensory ganglia

and periodically reactivates in peripheral tissues, local immune surveillance is essential for limiting viral spread and preventing recurrent lesion [49, 50]. Tissue-resident memory $CD8^+$ T cells are strategically positioned to provide this first line of defence, rapidly recognizing and controlling infected cells upon viral reactivation. While acyclovir effectively reduced disease severity and improved clinical outcomes in our model, our data suggest that early suppression of viral replication may simultaneously reduce signals required for optimal development of local resident-memory precursors. These findings do not diminish the clinical value of antiviral therapy but rather highlight the complex relationship between pathogen control and immune memory formation.

A limitation of the present study is that the analyses were performed during the post-acute phase of infection and therefore focused primarily on the early generation of TRM-like populations. Although the phenotypic markers evaluated are widely associated with tissue residency, the long-term persistence, functionality, and protective capacity of these cells remain to be determined. Future studies will be necessary to establish whether the reductions observed following antiviral treatment persist during the memory phase and whether they influence protection against subsequent viral reactivation or reinfection. In addition, the functional properties of these tissue-associated T cells will be further investigated through confocal microscopy and intracellular IFN-γ response analyses following ex vivo stimulation. For this purpose, genital tissue-derived T lymphocytes will be co-cultured with dendritic cells pulsed with the immunodominant HSV-2 $CD8^+$ T-cell epitope SSIEFARL, allowing the evaluation of antigen-specific effector responses and their relationship with the development and maintenance of local TRM populations.

Collectively, our findings support a model in which acute HSV-2 replication generates a local antigenic niche that promotes not only effector $CD8^+$ T-cell expansion but also the initiation of tissue-residency programs. By reducing viral replication, acyclovir diminishes the antigenic and inflammatory signals required for optimal TRM precursor formation, resulting in a reduced accumulation of $CD8^+$ T cells expressing residency-associated markers. These observations reinforce the concept that local antigen availability is a key determinant of tissue-specific immune memory development during HSV-2 infection.

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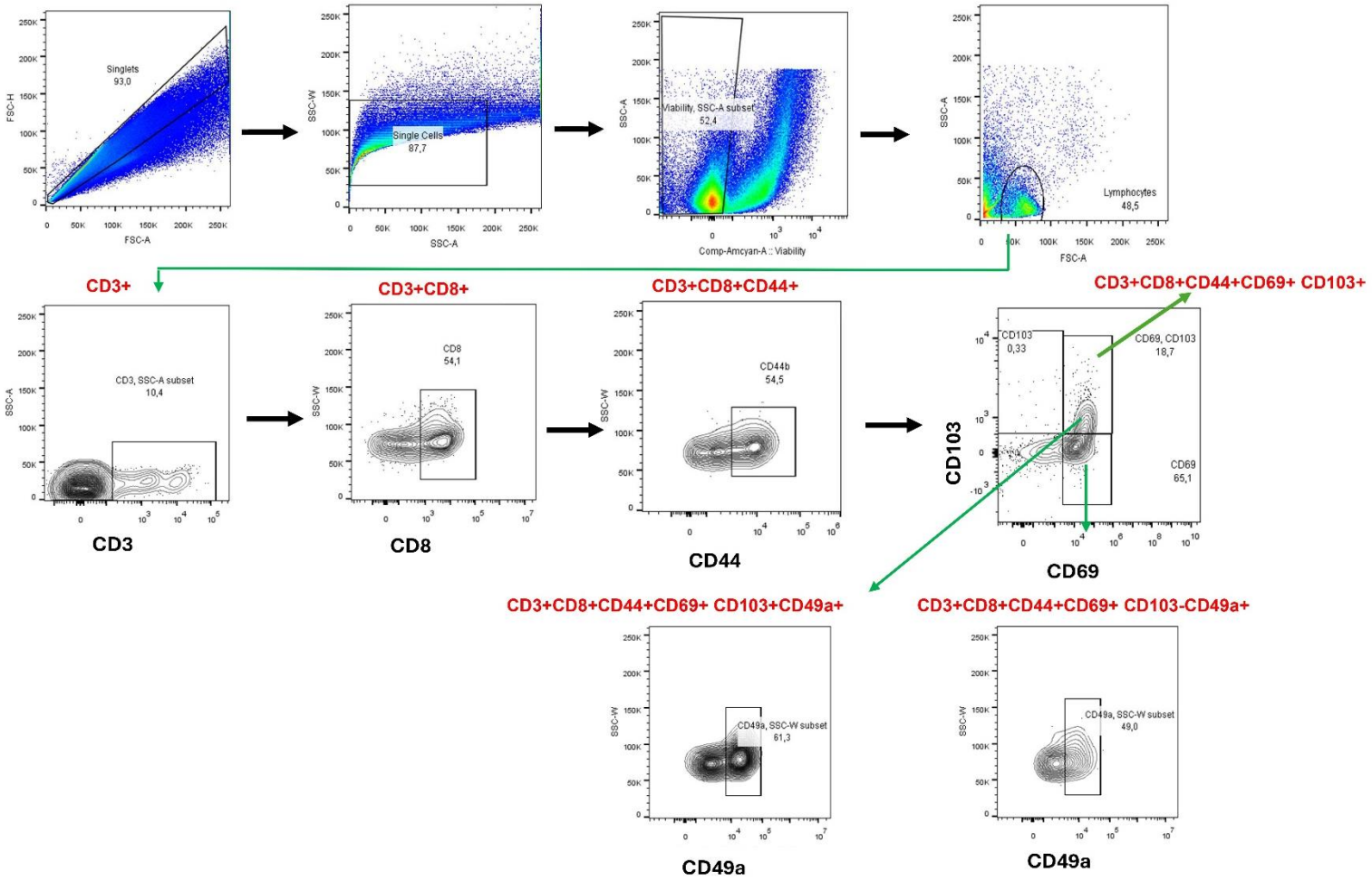
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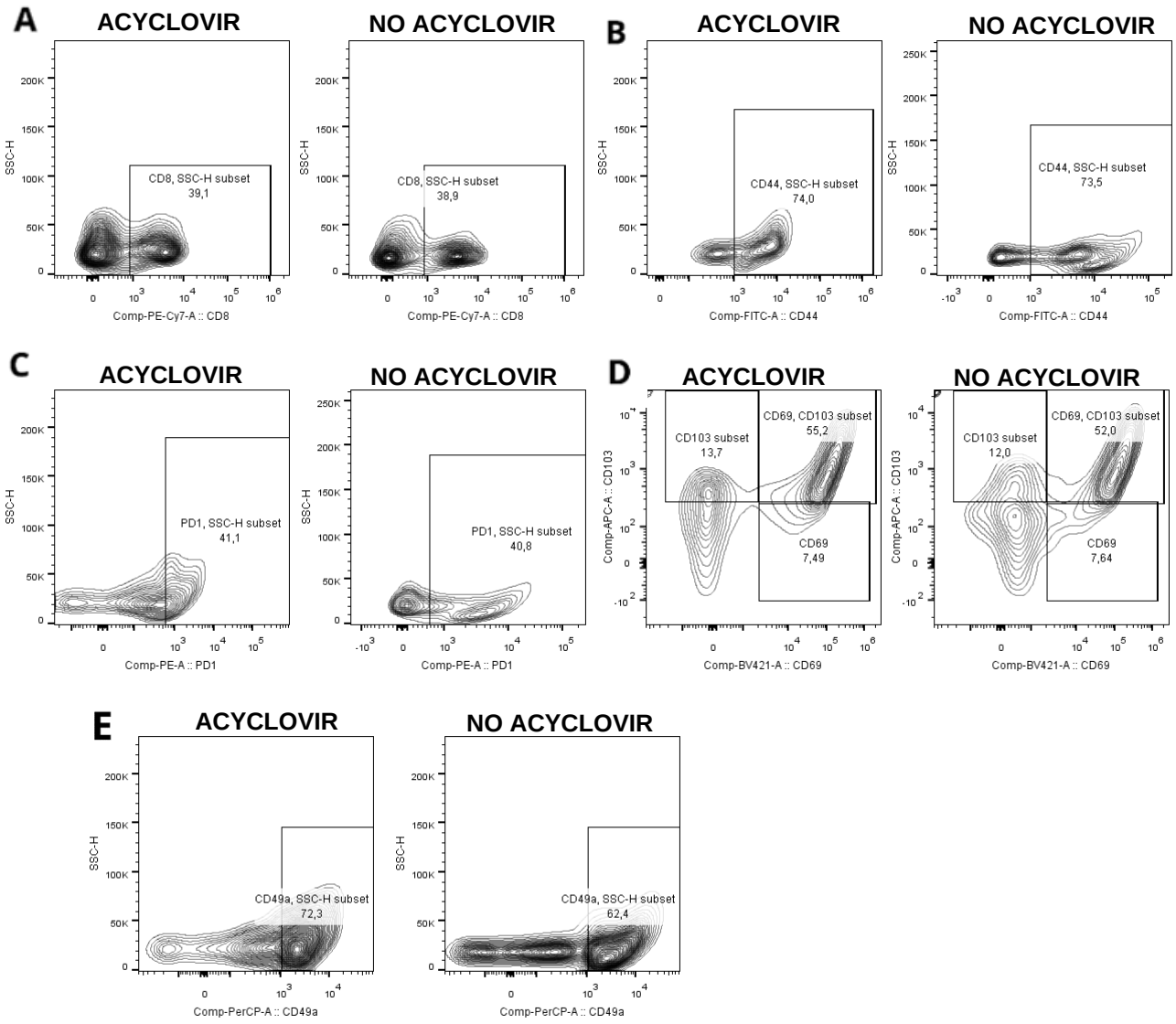
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## SUPLEMENTARY FIGURES



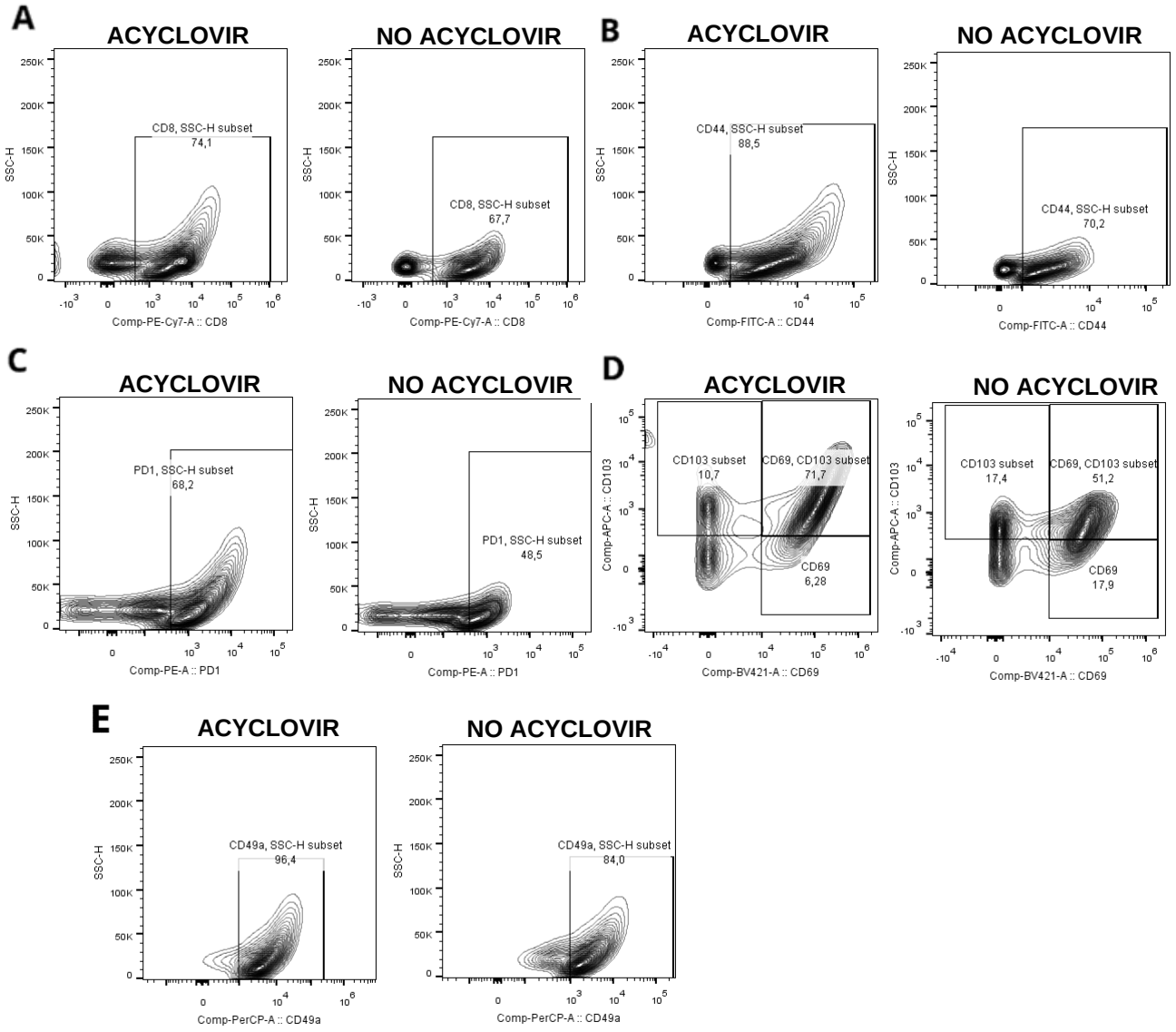

**Supplementary Figure 1 – Gate strategy.**

# SPLEEN




**Supplementary Figure 2 - The impact of acyclovir on the systemic immune response is limited in Spleen - Plots:** (A) Percentage of T $CD8^+$ lymphocytes in the spleen; (B) Percentage of T $CD8^+CD44^+$ lymphocytes; (C) Percentage of $CD8^+CD44^+PD1^+$ cells; (D) Percentage of $CD8^+CD44^+CD69^+CD103^+$ cells; (E) Percentage of $CD8^+CD44^+CD69^+CD103^+CD49a^+$ cells;

# LYMPH NODE




**Supplementary Figure 3 - The impact of acyclovir on the systemic immune response is limited in draining Lymph Node - Plots:** (A) Percentage of T $CD8^+$ lymphocytes in the draining lymph node; (B) Percentage of T $CD8^+CD44^+$ lymphocytes; (C) Percentage of $CD8^+CD44^+PD1^+$ cells; (D) Percentage of $CD8^+CD44^+CD69^+CD103^+$ cells; (E) Percentage of $CD8^+CD44^+CD69^+CD103^+CD49a^+$ cells;

## 5. CONCLUSÕES

Os resultados deste estudo demonstram que a resposta imune T CD8$^+$ na mucosa genital durante a fase aguda da infecção pelo HSV-2 é modulada pela carga viral inicial e pela disponibilidade de antígeno no tecido. Observou-se que diferentes doses de inóculo viral influenciam diretamente a magnitude da resposta imune local, impactando a frequência de linfócitos T CD8$^+$ ativados e a geração de células com fenótipo compatível com precursores de TRM, evidenciando o papel central do estímulo antigênico na programação inicial dessas populações.

Adicionalmente, os dados indicam que a redução da replicação viral, devido ao tratamento com Aciclovir, modula significativamente a resposta imune no sítio de infecção. Essa modulação se manifesta por uma diminuição na ativação de linfócitos T CD8$^+$ e na frequência de células expressando marcadores associados à residência tecidual, sugerindo que a redução precoce da carga antigênica pode impactar os processos iniciais de geração e estabelecimento de células TRM no tecido genital.

Em contraste, a resposta imune sistêmica apresentou alterações discretas, reforçando que a dinâmica observada é predominantemente local e dependente do microambiente tecidual. Esses achados sustentam a hipótese de que a disponibilidade de antígeno durante a fase aguda é um fator determinante na formação de precursores de TRM, sendo modulada tanto pela intensidade da infecção quanto por intervenções terapêuticas.

Dessa forma, este estudo contribui para a compreensão dos mecanismos iniciais que regulam a diferenciação de linfócitos T $CD8^+$ em direção ao fenótipo residente, destacando o papel da carga viral e do controle farmacológico da infecção nesse processo. Esses resultados têm implicações relevantes para estratégias terapêuticas voltadas ao controle de infecções por HSV-2, especialmente no que se refere à modulação da imunidade tecidual e à prevenção de episódios recorrentes.

## 6. CONSIDERAÇÕES FINAIS

Os achados deste estudo reforçam o papel central da disponibilidade de antígeno durante a fase aguda da infecção pelo HSV-2 como determinante da magnitude e da qualidade da resposta imune local, particularmente na geração de precursores de células T $CD8^+$ com características associadas à residência tecidual. A observação de que a modulação farmacológica da disponibilidade de antígeno, por meio do uso de aciclovir, está associada à redução na ativação de linfócitos T e na frequência de células com fenótipo compatível com TRM levanta uma questão relevante do ponto de vista imunológico: até que ponto a contenção precoce da carga viral, embora clinicamente benéfica, pode limitar sinais críticos necessários para o estabelecimento de uma imunidade tecidual robusta e duradoura.

Essa interpretação, no entanto, deve ser considerada com cautela. O delineamento experimental adotado restringe a análise à fase aguda da infecção, não permitindo inferir de forma direta se as alterações observadas na fase inicial se traduzem, de fato, em diferenças na formação, manutenção ou funcionalidade de populações de memória a longo prazo. Além disso, a definição de precursores de TRM baseou-se predominantemente em marcadores fenotípicos, os quais, embora amplamente utilizados, não são exclusivos desse tipo celular e podem refletir estados transitórios de ativação. A ausência de abordagens funcionais ou de rastreamento transcricional limita a capacidade de estabelecer relações causais mais robustas entre a modulação da carga antigênica e o destino dessas células.

Outro aspecto que merece consideração é a complexidade do microambiente tecidual, que envolve não apenas a presença de antígeno, mas também sinais inflamatórios, interações celulares e fatores locais que não foram diretamente avaliados neste estudo. Dessa forma, embora os dados apontem para um papel relevante da carga viral na programação inicial da resposta, é provável que a diferenciação de células TRM sofra influência da integração de múltiplos sinais, cuja contribuição relativa ainda precisa ser mais bem definida no contexto da infecção por HSV-2.

Neste sentido, os resultados apresentados devem ser interpretados como parte de um processo mais amplo de investigação, que terá continuidade no âmbito do Programa de Pós-Graduação em Patologia, com estudos em nível de doutorado sob a mesma linha de pesquisa em desenvolvimento no laboratório de Imunovirologia da UFCSPA. Trabalhos futuros serão direcionados à avaliação da fase de memória tardia, incluindo a análise da persistência, distribuição e funcionalidade de linfócitos T $CD8^+$ residentes em períodos prolongados após a infecção. Adicionalmente, será investigada a capacidade dessas células de responder a estímulos secundários, como sinais inflamatórios ou adjuvantes imunológicos, incluindo CpG, bem como o impacto do tratamento prévio com aciclovir na qualidade da memória estabelecida e na resposta a episódios de reativação viral.

Dessa forma, a continuidade deste projeto permitirá avançar na compreensão dos mecanismos que conectam os eventos iniciais da resposta imune à formação de memória tecidual funcional, contribuindo para uma visão mais integrada da imunidade contra o HSV-2. Esses desdobramentos são relevantes no contexto do desenvolvimento de estratégias terapêuticas, nas

quais o equilíbrio entre controle da replicação viral e adequada programação da resposta imune representa um grande desafio.

# 7. APÊNDICE I

## 7.1 Artigo publicado

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# Keeping it local: how CD8 TRMs regulate viral and cancer immunity

Luiz Rodrigues Junior[1,2,3]*, Cristina Bonorino[2], Alisson Felipe Haubert[1,3], Marvin Paulo Lins[4], Gabriel Pozo Pereira[2], Pedro R. Torres Romao[2] and Barry T. Rouse[5]

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The induction of immune responses in tissues and mucosa has emerged as one of the most promising strategies for the development of more effective vaccines and immunotherapies. In this context, $CD8^+$ resident memory T cells ($CD8^+$ TRM) have arisen as key players in local immune surveillance, acting persistently within non-lymphoid tissues. These cells represent a new and promising frontier in local immune responses and as potential clinical tools. $CD8^+$ TRM are being extensively investigated as therapeutic targets against viral infections and cancer, although their clinical applications have yet to be fully established. Understanding the molecular signals that regulate their generation, differentiation, maintenance, and activation is crucial for the precise targeting of their immune functions. This review explores the main mechanisms involved in the formation and maintenance of $CD8^+$ TRM, from the strength of MHC: TCR interactions to the coordinated role of cytokines, chemokines, and transcription factors in tissue retention and the expression of markers such as CD69, CD103, and CD49a. By integrating this knowledge, we discuss strategies to manipulate these pathways with the goal of developing more effective vaccines and personalized therapies based on resident memory T cells. We also examine how these molecular signals and pathways, either independently or in combination, can be explored both in the fight against viral infections and cancer, and in identifying $CD8^+$ TRM predictive biomarkers for response to anticancer immunotherapies across various tumor types.

KEYWORDS

**CD8, TRM, virus, cancer, lymphocyte**

# 1 Introduction

The idea that protection against a disease could be induced by inhaling ground up scabs from those that recovered from smallpox came from the Chinese more than 1000 years ago (1). This might be construed as the dawn of mucosal immunology. However, a more mechanistic understanding of local immunity was established in the 1960 with the demonstration that local immunization induced a unique immunoglobulin type referred to a secretory IgA (2). Subsequent studies showed that the greater efficacy of the oral Sabin polio vaccine to protect against enteric poliovirus infection was explained by its ability to induce secretory IgA at mucosal sites, which was absent following immunization with inactivated Salk vaccines (3). These observations on mucosal immunity focused on antibody mediated immune protection, but as we now know, the resolution of many infections and some cancers is carried out mainly by components of cellular immunity, particularly T cells.

Resident memory T (TRM) cells were initially identified as a heterogeneous population, both in terms of surface molecule expression and functional properties. Further complexity was revealed when it was demonstrated that T cells residing at tissue entry sites can be distinguished from memory T cells in systemic and lymphoid compartments based on membrane markers, transcriptional profiles, and cytokine responsiveness (4–6). Multiple studies have provided evidence that the signals governing TRM generation, maintenance, and function can vary not only between different tissues but also between distinct sites within the same tissue. This highlights the complexity and tissue-specific nature of the TRM differentiation program (7, 8). For instance, skin and intestinal TRMs heavily depend on TGF-β for their development and maintenance. In the intestine, however, an additional pathway involving retinoic acid signaling also contributes to TRM generation. While skin TRMs primarily rely on both TGF-β and retinoic acid, intestinal TRMs can utilize either factor. Nonetheless, when TGF-β is available, it remains the dominant driver of intestinal TRM formation (9). These findings underscore that TRM differentiation is governed by intricate, context-dependent molecular cues that are still being elucidated, particularly regarding how local signals and T cell receptor (TCR)-mediated pathways cooperate to define their activation and persistence.

The concept of TRM cells originated from seminal studies demonstrating that non-migratory T cells persist long-term within peripheral tissues following herpes simplex virus (HSV) skin infection, as shown through transplantation experiments (10). Similarly, intestinal graft models revealed that memory T cells embedded within the small intestinal epithelium remain locally confined and do not recirculate (11). Further insights came from parabiosis experiments, which showed that TRM cells not only persist at the initial site of infection but also disseminate throughout the entire skin surface, maintaining long-term residency (12). Phenotypically, TRM cells are defined by the expression of key integrins such as CD69, CD103, and CD49a, which orchestrate their retention and functional specialization within non-lymphoid tissues (13, 14). CD69 antagonizes sphingosine-1-phosphate receptor 1 ($S1P_1$), thereby preventing tissue egress (15). CD103 (αE integrin) binds to E-cadherin on epithelial cells, promoting localization and anchorage within barrier tissues (16). CD49a (α1 integrin), which interacts with collagen IV, is associated with enhanced cytotoxic activity and long-term survival within tissue niches (17). Collectively, these surface molecules delineate the TRM phenotype and underlie their central functions, continuous immune surveillance and the rapid initiation of localized effector responses upon reinfection, thereby providing potent, site-specific protective immunity. Altogether, the combination of tissue residency and expression of adhesion and retention molecules defines the unique identity and immunological role of TRM cells.

Over the years, it has become evident that the tissue-surveilling capacity of TRM cells surpasses that of central memory (TCM) and effector memory (TEM) T cells in providing rapid and effective immunity at barrier sites. Studies using murine models of viral skin and lung infections demonstrated that TRM cells can promptly produce effector molecules locally, enabling them to respond more rapidly to pathogen re-exposure within the tissue compared with their circulating counterparts (12, 18). These findings have positioned TRMs as promising targets for immunotherapeutic interventions, particularly in cancer and chronic viral infections, and have also highlighted the need to evaluate vaccines based on their capacity to elicit tissue-resident responses. Nevertheless, several emerging new concepts in the TRM field require further evaluation before their clinical application becomes feasible. In this context, the present review examines the mechanisms involved in TRM induction and function across infection and cancer models, aiming to support the development of strategies with real translational potential.

Furthermore, this article provides a comprehensive synthesis of the molecular and transcriptional programs underlying $CD8^+$ TRM differentiation. We link TCR signal strength with local cytokine and chemokine cues, illustrating how these pathways converge to shape TRM generation. By comparing antiviral and tumor settings, we reveal that similar molecular circuits govern TRM development in both contexts yet display remarkable plasticity according to the tissue microenvironment. A key contribution of this work is the discussion of how TCR–peptide–MHC affinity influences TRM formation and integrates with additional local signals to convert effector $CD8^+$ T cells into long-lived residents. By positioning TRMs as pivotal mediators of local immunity, we highlight potential strategies to modulate TRM-inducing signals—bridging mechanistic insights from infection and cancer toward translational applications.

# 2 Lessons learned from the classic subpopulations of memory T cells

Memory T cells are essential to long-term protection against infectious diseases and contributes for antitumor immunity. Their generation and maintenance have been extensively investigated, particularly in the settings of viral infections and cancer (19–21). Upon recognition of antigens presented by Major Histocompatibility Complex class I (MHC I) molecules on Antigen-Presenting Cells

(APCs), along with co-stimulatory and cytokine signals, naïve $CD8^+$ T cells become activated and undergo clonal expansion and differentiate into effector cells that produce IL-2 and mediate pathogen clearance. Following antigen elimination, most of these cells, approximately 90%, undergo apoptosis during the contraction phase, while a minority survive and initiate the memory differentiation program. This process gives rise to two major subsets: short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) (22). The developmental bifurcation between these subsets is governed by distinct transcriptional and signaling programs. MPECs, which eventually give rise to long-lived memory T cells, integrate multiple cues such as weaker TCR stimulation (23), cytokines like IL-15 (24), and transcriptional regulators including Bcl-6 (25) and Eomes (26), in addition to epigenetic mechanisms that stabilize the memory gene expression profile (27). The transcription factors Forkhead box protein O1 [FoxO1] (28) and T cell factor 1 [Tcf-1] (29) are central to this process: both are highly expressed in naïve $CD8^+$ T cells, transiently downregulated during effector differentiation, and subsequently re-expressed as cells transition toward the memory state. This dynamic regulation indicates that the transcriptional circuitry underlying memory formation is preconfigured in naïve cells, temporarily suppressed during effector commitment, and reactivated to sustain longevity and homeostatic responsiveness. In contrast, SLECs fail to reinitiate this memory-associated program, resulting in terminal differentiation and apoptosis (30). Consequently, only a small fraction—approximately 5%—of the activated $CD8^+$ T cell population persists as long-lived memory cells (31). A schematic overview of the memory T cell subsets generation and maintenance is present in Figure 1.

The maintenance and differentiation of TEM and TCM cells rely heavily on the expression of cytokine receptors such as IL-7, IL-15, and IL-21, features that have been extensively characterized (32, 33) which dependent on the expression of CD122 (IL-2/IL-15 receptor β chain) and CD132 (common gamma chain, γc) (34, 35).Traditionally, memory T cell subsets have been classified based on their tissue localization, surface marker expression, and functional attributes. Following differentiation, TCMs predominantly reside in lymphoid tissues, whereas TEM cells circulate through the blood, secondary lymphoid organs, and peripheral tissues. TCMs are marked by the expression of lymph node homing receptors CD62L and CCR7, which are absent in TEM cells (36, 37).

Following antigen clearance, memory T cells initiate a developmental program that supports continued clonal expansion. Remarkably, these cells can undergo up to eight rounds of division in the absence of cognate antigen, a process sustained by homeostatic signals from γc receptor cytokines (38–40). Compared to their naïve counterparts, memory T cells possess

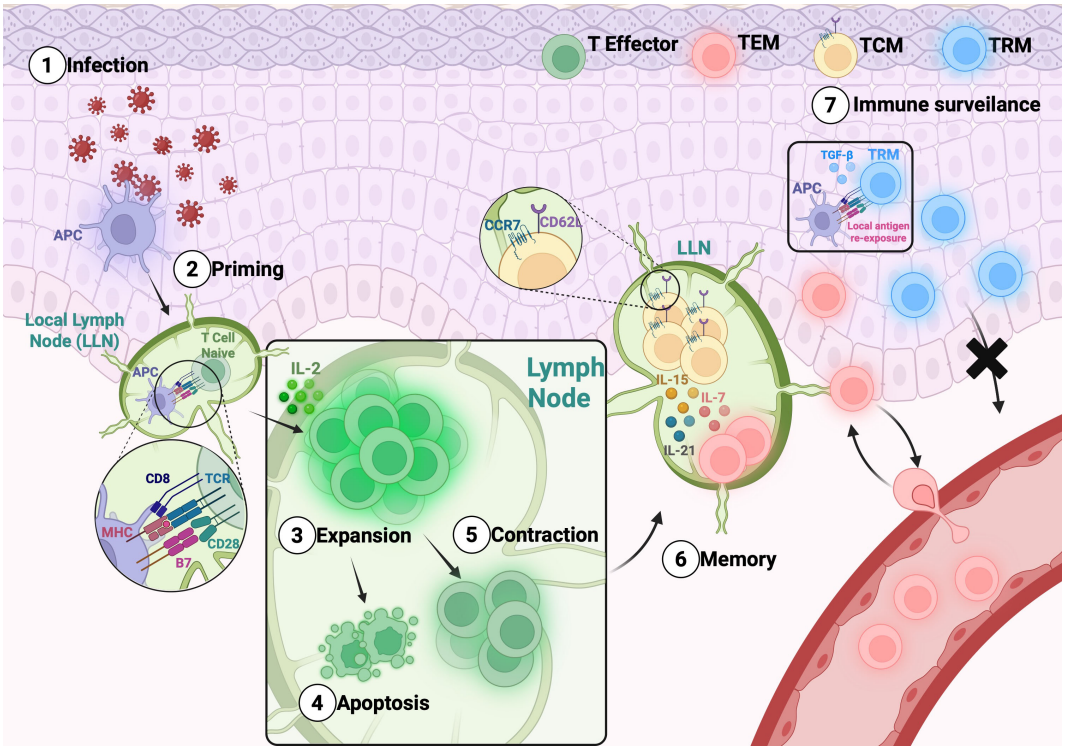



FIGURE 1
General overview of the memory T cell subsets generation and maintenance:. After infection (1), the differentiation of memory T cells includes a priming phase (2) in the LN. After expansion (3) the antigen is cleared, and part of the T cells enter in apoptosis (4) and part survive – contraction phase (5). Further, T cells differentiate into TCM, which circulate between the blood and secondary lymphoid tissues, and TEM, which exit the LN to enter the circulation and peripheral tissues (6). TRM cells are generated from T effector differentiation that migrate to the tissue and remain there to rapidly face antigen challenge. TRMs can recognize antigens within tissues and, upon stimulation with cytokines such as TGF-β, can induce or enhance the expression of residency markers (7).

lower activation thresholds, requiring reduced antigen levels and shorter TCR signaling durations to become fully activated (41). Transcription factors (TF) play a central role in the rapid recall capacity of memory $CD4^+$ T cells. While T-bet is indispensable for IFN-γ expression in naïve T cells, memory $CD4^+$ T cells can rapidly produce this cytokine without upregulating T-bet. Instead, this response is linked to enhanced nuclear translocation of NFκB p50, suggesting that increased accessibility of promoter regions allows more efficient NFκB recruitment, thereby driving accelerated gene expression during recall responses (42). The molecular mechanisms governing the induction and maintenance of T cell memory have been thoroughly reviewed elsewhere (43–46).

TRM cells arise within a signaling framework partially shared with other memory T cell subsets but acquire unique features due to their differentiation and function across diverse tissues. Their development is shaped by local environmental cues, including variations in antigen availability, differential expression of adhesion molecules, distinct cytokine and chemokine responsiveness, and tissue-specific transcriptional programs. Consequently, the biology of TRMs diverges substantially from that of other memory T cell subsets, and their presence is strongly associated with immune protection. These characteristics critically influence T cell responses in both viral infections and cancer.

## 3 What drives a memory $CD8^+$T cell to become a TRM?

TRM cell differentiation follows a distinct pathway compared to other memory T cell subsets. Evidence indicates that $CD8^+$ effector T cells, once primed in local lymph nodes, acquire TRM lineage traits and subsequently migrate to peripheral tissues through the bloodstream (13). As the infectious process begins to resolve, a subset of these pre-committed effector cells persists at the site of inflammation and differentiates into $CD8^+$ TRM cells under the influence of local environmental cues, including homing molecules, inflammatory cytokines, and chemokines (47, 48). For instance, during viral infections, $CD8^+$ T cells expressing P-selectin ligands migrate along CXCL10 gradients produced by endothelial cells in response to type I interferons (49). Like TEM and TCM, it is now understood that primary TCR signaling in the lymph node (50) together with local inflammatory signals, cytokines, and chemokines, plays a sequential and collaborative role in the generation, proliferation, and maintenance of TRMs (51). However, the precise identity and timing of these signals vary across tissues and remain incompletely understood. The following section explores the molecular and environmental factors that guide effector $CD8^+$ T cells toward tissue-specific TRM differentiation.

### 3.1 The influence of TCR signaling to form TRMs

TCR signaling plays a crucial role in inducing the expression of adhesion molecules characteristic of TRMs. In a mouse model of intranasal viral-vectored immunization using a *Mycobacterium tuberculosis* antigen, antigen-specific $CD8^+$ T cells began expressing CD103 shortly after entering lung tissue, and its expression increased during the contraction phase (52). A similar pattern was observed in mice infected intranasally with influenza virus, where effector $CD8^+$ T cells recognizing their cognate antigen in the lung upregulated CD103 and maintained CD69 expression (53). Consistent findings in intestinal (54) and skin (50) models confirm that local antigen recognition is essential for efficient TRM formation. In a mouse kidney transplant model, the persistence of antigens presented by recipient-derived dendritic cells was also crucial for maintaining TRM stability and function (55). Likewise, in viral infection models, antigen availability determined TRM differentiation and longevity. For instance, intracerebral delivery of vesicular stomatitis virus (VSV) antigens was required to sustain CD69 and CD103 expression on brain $CD8^+$ T cells, with $CD103^+$ clusters persisting up to 30 days post-infection, even after viral clearance (56).

Although cognate antigen is essential for TRM formation, the recruitment of antigen-specific circulating effector T cells into tissues can also be driven by local inflammatory cues. In this context, tissue-derived cytokines and chemokines promote TRM differentiation (48). This was demonstrated in a skin infection model with HSV, where inflammation induced solely by the contact sensitizer 2,4-dinitrofluorobenzene (DNFB) was sufficient to recruit previously generated antigen-specific $CD8^+$ T cells, which subsequently expressed CD103 and differentiated into TRMs (57). In the same model, broader tissue protection mediated by TRMs was achieved through localized antigenic restimulation. Repeated antigen exposures in distinct skin regions generated spatially dispersed TRMs, whereas a single high-dose injection at one site led to TRM formation restricted to that specific area (58). Conversely, the generation of pulmonary $CD8^+$ TRMs, as well as the differentiation of circulating effector T cells, critically depends on local antigen recognition within the lung. Thus, although inflammatory cues such as cytokines and chemokines can recruit circulating effector $CD8^+$ T cells to the pulmonary tissue, these signals alone are insufficient to induce TRM differentiation. Only upon cognate antigen encounter *in situ* do these effectors cells acquire the transcriptional and phenotypic features characteristic of long-lived TRMs.

The affinity between the MHC I–peptide complex and the T cell receptor is a key determinant guiding effector T cells toward the TRM phenotype. Frost et al. (59) demonstrated this relationship by isolating memory cells from the spleen, brain, and kidney of mice intracerebrally infected with polyomavirus, a virus that preferentially replicates in the kidney. Upon ex vivo antigen challenge, TRMs from the brain and kidney displayed approximately 20-fold higher peptide affinity compared to splenic memory cells, suggesting that TRMs originate from high-affinity effector cells that infiltrate non-lymphoid tissues during acute infection. This finding implies that TRMs may possess distinct TCR repertoires compared with other antigen-specific memory subsets. Conversely, in a systemic polyomavirus infection model, higher numbers of brain $CD8^+$ TRMs were observed in groups

primed with subdominant epitopes that generated weaker TCR stimulation (60). Likewise, in a murine influenza model, $CD8^+$ T cells with lower TCR affinity exhibited a competitive advantage in forming lung TRMs, although affinity did not appear to influence their persistence or functional quality (61). Finally, in a study using adoptive transfer of transgenic $CD8^+$ T cells specific for lymphocytic choriomeningitis virus (LCMV), followed by ear skin infection with Vaccinia virus expressing the LCMV peptide GP33, the presence of cognate antigen was required for IFN-γ production and TRM formation. When Vaccinia variants expressing a modified SIINFEKL peptide with reduced affinity for the OT-I TCR were used, the strength of TCR engagement was shown to modulate both IFN-γ expression and the transcriptional programs underlying TRM differentiation (62).

The contribution of cognate antigen to TRM-mediated protection has been demonstrated across several models, including malaria vaccination targeting the liver (63, 64) hepatitis C mouse models (65), and the female reproductive tract during genital HSV-2 infection (66). Together, these studies reinforce that antigen presentation and the strength of TCR engagement are central to $CD8^+$ T cell differentiation into TRMs. Nevertheless, the precise influence of peptide-MHC: TCR affinity on TRM generation and tissue establishment remains incompletely defined and appears to vary according to factors such as infection type and immunization route. Table 1 summarizes representative studies highlighting this variability in TCR interaction strength and its relationship with TRM formation.

## 3.2 The expression of functional TRMs markers for tissue lodging and location

Beyond antigen-driven TCR signaling, $CD8^+$ TRM lymphocytes depend on specific molecules that support their long-term residence within tissues (70). Upon entry, pre-committed effector $CD8^+$ T cells activate transcriptional programs that consolidate the TRM phenotype, notably through the expression of adhesion and retention molecules (71, 72). Among these, CD69, CD103, and CD49 have been widely used as defining markers, although their functional relevance varies across tissues and microenvironmental contexts (73–75).

CD69 is strongly upregulated upon TCR-mediated lymphocyte activation (76) and plays a key role in tissue retention, metabolism, and maintaining an activated T cell phenotype (77). Mechanistically, CD69 binds in cis to sphingosine-1-phosphate receptor 1 (S1P1R) on the T cell membrane, triggering receptor internalization and degradation (78). Without S1P1R, T cells cannot respond to circulating sphingosine-1-phosphate (S1P), resulting in their retention within tissues (15, 79). Through this mechanism, early CD69 expression effectively inhibits T cell egress and promote TRM residency.

CD49a (very late antigen 1, VLA-1; α1β1 integrin) binds collagen IV in non-lymphoid tissues (80, 81). Its expression occurs early during $CD8^+$ TRM generation. For instance, following mouse intranasal immunization with a viral-vectored *M. tuberculosis* antigen, most antigen-specific $CD8^+$ T cells

TABLE 1 Studies on peptide–MHC: TCR affinity in the format ion of CD8 TRMs.

| Model/Tissue | How affinity/Signal was measured or manipulated | Main finding | Reference |
|---|---|---|---|
| Mouse — circulating $CD8^+$ memory (not specific to TRM) | Genetic/pharmacologic reduction of TCR signaling and antigen affinity variation | Lower signaling/affinity can favor memory — reduced TCR signaling accelerated and increased $CD8^+$ memory generation. | (23) |
| Mouse — brain & kidney (Mouse polyomavirus infection) | 2D affinity (micropipette), tetramer binding | High affinity favored — TRM displayed ~20× higher TCR affinity than splenic memory cells. | (61) |
| Mouse — brain (Polyoma virus infection) | Varied antigenic stimulation strength | Lower affinity favored/better function — suboptimal stimulation recruited more functional brain TRM; strong TCR signals impaired memory development. | (62) |
| Mouse — lung (Influenza A infection) | OT-I epitope variants with high vs low TCR affinity | Lower/intermediate affinity favored— pulmonary TRM formation was higher with weaker TCR stimulation; strong signals reduced TRM generation. | (63) |
| Mouse — skin/ear (Vaccina virus model) | Manipulated signal intensity; readout: Blimp1, CXCR6, S1PR1 | Mixed/context dependent. Stronger signaling promoted $CXCR6^+$ retention and inhibited egress via S1PR1; both high and moderate signals contributed differently. | (65) |
| Mouse — brain (chronic infection with Toxoplasma gondii) | Comparison among T cell clones of different TCR affinities | High affinity favored — higher affinity clones trafficked better to the brain and generated more $CD103^+$ TRM. | (67) |
| Review — lung/trachea (human and mouse) | Compilation of multiple experimental studies | Contextual/heterogeneous — affinity/signal impact varies by tissue and microenvironment; in lungs, lower affinity can sometimes favor TRM. | (68) |
| Review — respiratory tract | Review of multiple models and affinity assessments | Context-dependent — lung microenvironment and inflammation modulate how TCR affinity/signal strength shapes TRM formation. | (69) |

Mouse polyoma virus infection (61), Toxoplasma gondii infection (67), Vaccinia virus model (65), Lung Influenza infection (63), Mouse polyomavirus infection in brain (62), Listeria monocytogenes strain carrying ovalbumin peptides (23), Review in lung TRMs (68), TRM in respiratory virus infection (69).

arrived in the lung already expressing CD49a, with expression enriched during contraction and memory phases (52). Comparable early CD49a expression was noted in a skin HSV-1 infection model. Interestingly, in both studies, CD49a was shown not to be essential for the generation of $CD8^+$ TRM lymphocytes in the tissue, as these cells arrive already expressing this marker. Although CD49a is not required for TRM generation, it correlates with IFN-γ production by skin TRMs during HSV infection and contributes to their maintenance and effector function within tissues (82).

CD103 (αEβ7 integrin) is a hallmark marker expressed on $CD8^+$ TRMs in infected tissues and certain tumors (83, 84). This integrin binds to E-cadherin, which is expressed on epithelial cells (85). In human tumors such as glioblastoma and breast cancer, elevated CD103 expression correlates with better prognosis, likely due to enhanced $CD8^+$ TRM infiltrations (86–88). CD103 expression on effector $CD8^+$ T cells is regulated by TGF-β. In viral infections, TGF-β promotes CD103 upregulation during the contraction phase in skin and lungs, aiding TRM differentiation in mouse (55, 89). Similarly, studies in a lung tumor (90) and HPV tumor models (91) have also shown that TGF-β induces the upregulation of CD103 on the surface of $CD8^+$ T lymphocytes, contributing to the formation of TRMs. CD103 is regarded as a reliable molecular marker for the characterization of $CD8^+$ TRMs, particularly those residing in epithelial tissues. Together with CD103, the identification of TRMs is often further supported by the co-expression of CD69 and CD49a (70).

Despite frequent use of CD69, CD103, and CD49a to identify $CD8^+$ TRMs, their expression is neither universal nor uniform, varying with tissue environment and inflammation. For example, CD69 is essential for TRM formation in the kidney, where its enforced expression promotes residency, but dispensable in the small intestine, illustrating tissue-specific requirements (92). Similarly, CD103 and CD49a expression depend on local cytokines and cell interactions such as TGF-β and integrin signaling and may be absent in certain tissues or disease contexts (93). These findings highlight TRM phenotypic heterogeneity and underscore the importance of tissue and disease-specific considerations in TRM characterization.

Altogether, integrin's receptors seem to act in concert to lodge $CD8^+$ T cells that are becoming TRM cells in their respective tissue - be it in lung, kidney (94), brain (95), intestine (96), skin (97), and female reproductive tract (98). However, we still know relatively little about the identity of site-specific molecules that guide TRM cells to reside in one tissue over another. Despite growing evidence of tissue-specific modulators influencing TRM marker expression, the precise molecules and mechanisms governing site-selective TRM localization remain poorly defined, warranting further investigation.

## 3.3 The role of cytokines in controlling TRM dynamics

Early studies on mouse memory $CD8^+$ T lymphocytes suggested that their long-term maintenance depends less on continuous TCR stimulation and more on homeostatic signals (99–101). This paradigm is similarly observed for $CD8^+$ TRMs, which appear to rely minimally on persistent antigen exposure for their maintenance. However, the homeostatic mechanisms governing TRMs may diverge from those regulating other memory subsets. Unlike lymphoid organs, barrier tissues are frequently exposed to environmental antigens and inflammatory stimuli, which generate unique local cues that support TRM survival and persistence.

Among these tissue-specific factors, TGF-β has emerged as a pivotal cytokine orchestrating the differentiation, retention, and long-term maintenance of $CD8^+$ TRMs in epithelial barrier tissues by inducing key retention molecules such as CD103 and CD69 that inhibit lymphocyte egress (102, 103). In LCMV infection models, effector $CD8^+$ T cells migrate to the intestinal mucosa where TGF-β drives CD103 expression independently of ongoing antigen stimulation, highlighting its essential role in imprinting tissue residency post-pathogen clearance. Furthermore, ex vivo stimulation of splenic $CD8^+$ T cells from LCMV-infected mice with TGF-β combined with IL-33 or TNFα enhances the expression of TRM-associated markers including CD69 and Ly6C, reinforcing the interplay between cytokines in TRM programming (104). Importantly, TGF-β's role extends beyond the intestine, controlling $CD8^+$ TRM maintenance in diverse tissues such as skin (89), kidney (105) and genital tract (91) in mouse models, indicating a conserved mechanism by which TGF-β integrates environmental signals to establish a stable TRM phenotype that ensures effective localized immune surveillance.

Interleukin-15 (IL-15) signaling via its receptor (IL-15R) is essential for the survival and homeostatic proliferation of $CD8^+$ TRMs, enabling their long-term persistence similarly to TEM cells (13, 89). Antigen-specific $CD8^+$ TRMs in tissues such as liver, kidney, skin, and salivary glands actively proliferate in response to IL-15 stimulation (106). In contrast, TRMs in certain sites like the small intestine, pancreas, and female reproductive tract show a reduced dependence on IL-15 despite retaining responsiveness, suggesting that local cytokine milieu distinctly influences TRM homeostasis (107). The balance between IL-15 and interleukin-7 (IL-7) signaling further fine-tunes TRM adaptation, notably, high levels of both cytokines lead to downregulation of IL-7 receptor alpha (IL-7Rα), whereas IL-7Rα is upregulated when IL-15 is scarce (108). This reciprocal regulation exemplifies a sophisticated homeostatic network enabling TRMs to tailor their survival strategies according to tissue-specific cytokine availability. Overall, IL-15 functions as a central, yet context-dependent, regulator of $CD8^+$ TRM persistence and self-renewal across non-lymphoid tissues.

Beyond IL-15 and IL-7, additional cytokines contribute to TRM maintenance and function, often in a tissue-specific manner. For example, in murine models of pulmonary infection, alveolar macrophages cross-present antigens to $CD8^+$ T cells while producing IL-18, which promotes lung TRM differentiation. In this context, mice deficient in the IL-18 receptor display impaired induction of $CD103^+$ antigen-specific $CD8^+$ T cells in the lung, suggesting that IL-18 indirectly supports TRM development by promoting $CD4^+$ Th1 cell differentiation and shaping the local cytokine milieu (74). Similarly, IL-21 partially regulates the

differentiation of intestinal $CD8^+$ effector T cells into TRMs, as shown in mixed bone marrow chimera models containing IL-21 receptor-deficient and wild-type cells (109). In the brain, increased IL-21 receptor expression on $CD8^+$ T cells during murine polyomavirus infection correlates with higher CD103 levels and enhanced TRM formation (110). Additionally, IL-33 has emerged as another cytokine with a crucial role in sustaining intestinal $CD8^+$ TRMs, although the precise mechanisms remain under investigation (111).

In summary, cytokines are fundamental regulators of $CD8^+$ TRM dynamics, influencing their differentiation, maintenance, and functional adaptation within diverse tissue microenvironments. Key cytokines such as TGF-β and IL-15 coordinate a delicate balance between tissue retention, homeostatic proliferation, and survival, while others, including IL-7, IL-18, IL-21, and IL-33, modulate TRM biology in a tissue-specific manner. Elucidating these cytokine-driven pathways will be critical for the development of targeted immunotherapies and vaccination strategies aimed at harnessing or enhancing tissue-resident immune memory.

## 3.4 Chemokines coordinate the migration and tissue distribution of TRMs

While cytokines are central to the differentiation and maintenance of $CD8^+$ TRMs, chemokines and their receptors play an equally critical role in shaping the tissue-specific localization, migratory behavior, and functional adaptation of these cells. Chemokine signaling is essential not only for recruiting effector T cells to non-lymphoid tissues but also for orchestrating their retention, positioning, and morphological transformation into TRMs.

The skin provides a well-characterized model of how chemokine-mediated cues drive TRM development and function. As a barrier tissue with constant environmental exposure, it establishes a specialized microenvironment where TRMs intercalate among keratinocytes and adopt a dendritic-like shape that optimizes local immune surveillance (112). The expression of chemokine receptor CXCR6 is required for $CD8^+$ T cells to differentiate into TRMs and acquire this morphology, facilitating their integration among keratinocytes (113). Among these, the CXCL16–CXCR6 interaction stands out as a key axis promoting epidermal TRM retention and survival (101). In parallel, CCR10 and its ligand CCL27, produced by keratinocytes, mediate skin-specific homing of T cells (114). Collectively, these chemokine pathways ensure proper TRM localization and persistence, supporting rapid and effective cutaneous immune protection.

Beyond tissue retention, chemokines play a pivotal role in guiding the initial migration of effector $CD8^+$ T cells from the circulation into peripheral tissues (115). Once within the tissue, the pre-committed effector cells can differentiate into TRMs, even in the absence of cognate antigen recognition, a process known as local heterotypic differentiation (116). One of the earliest detailed demonstrations of this mechanism arose from studies of viral infection in the female reproductive tract. Upon secondary antigen encounter, resident $CD8^+$ TRM cells rapidly produced interferon-gamma (IFN-γ), along with the chemokines CXCL9 and CXCL10, which acted on local endothelial cells to modify the vasculature and facilitate the recruitment of additional memory T cells from the circulation. These newly recruited cells, drawn into the inflamed tissue microenvironment, subsequently differentiated into TRMs and expanded the local pool of tissue-resident memory cells (117). This dynamic indicates that the exclusion of memory T cells from certain non-lymphoid compartments is not absolute, but rather context-dependent, and can be transiently overridden when local TRMs initiate a chemokine-driven "alarm" in response to antigen recognition. Such mechanisms likely serve to accelerate the recruitment of pathogen-experienced T cells to sites of reinfection, thereby enhancing immune responsiveness. Supporting this concept, additional studies in human skin have shown that expression of the chemokine receptor CCR8 on $CD8^+$ T cells correlate with the expression of canonical TRM markers, including CD103 and CD69, as well as with gene expression profiles associated with tissue residency (118). These findings underscore the close link between chemokine receptor expression, migratory cues, and the establishment of the resident memory phenotype in peripheral tissues.

In the central nervous system (CNS), chemokine-driven recruitment of $CD8^+$ T cells are often associated with inflammation or tissue damage rather than protective immunity. During aging, the basal release of chemokines—including CCL5, CCL11, CXCL9, and CXCL10 promotes the infiltration of $CD8^+$ T cells into the CNS. This process appears to be largely antigen-independent and stochastic, often associated with ischemic injury. Once inside the CNS, these infiltrating cells can give rise to a population of "age-associated" $CD8^+$ TRMs characterized by the expression of CD103, CD69, and PD-1 (119). In contrast, in mucosal sites chemokine signaling can also sustain protective TRM responses. In the genital mucosa, for instance, HSV-1 infection does not always lead to lesion formation; instead, resistance correlates with local CXCL17 expression and the recruitment of $CD8^+$ T cells bearing its receptor, CXCR8. These $CD8^+CXCR8^+$ cells acquire a TRM phenotype and confer enhanced protection against recurrent herpes lesions (120).

Compared to canonical TRM markers CD103 and CD69, the expression of chemokines and their receptors is more tightly associated with the anatomical localization and functional specialization of TRMs. Some chemokine receptors primarily guide the localization of TRMs within tissues, while others directly influence their activation, morphology, or survival. For instance, in the salivary glands, sites of cytomegalovirus infection and replication, CXCR3-mediated $CD8^+$ T cell migration appears to be an intrinsic property of the tissue rather than a response to infection. The salivary gland constitutively recruits $CD8^+$ T cells in a CXCR3-dependent manner and is capable of inducing and sustaining TRM populations even in the absence of infection, antigen, or inflammation (121). Moreover, the CXCR3–CXCL10 signaling axis is essential for TRM recruitment and accumulation (122), while the CXCL16–CXCR6 pathway has been shown to promote the retention of $CD8^+$ TRMs in barrier tissues such as

the skin and lungs (123, 124). Collectively, these findings highlight that chemokines function not merely as passive recruiters of T cells but as active regulators in shaping TRM functions in the tissues. They mediate the initial recruitment of effector cells, modulate their retention, influence the expression of residency-associated markers, and tailor TRM functionality according to the specific demands of the local tissue microenvironment. As such, chemokines are indispensable components of the tissue residency program, acting in concert with cytokines and other local signals to establish robust, site-specific immune memory. In Figure 2, we illustrate the principal cytokines, chemokines, and integrins involved in $CD8^+$ TRM formation and maintenance across the most studied tissues (skin, gut, and lung).

## 3.5 Signals for TRM differentiation are driven by the kinetics of transcription factors in tissues

$CD8^+$ TRM cells exhibit tissue-specific transcriptional programs that are largely established during the peak of the effector T cell response (125, 126). Upon activation within non-lymphoid tissues, effector $CD8^+$ T cells initiate a transcriptional cascade that programs them toward a resident memory phenotype (13, 127). For example, in the skin, only effector cells that activate the appropriate gene expression profile and receptor repertoire adopt a dendritic morphology and become functional TRM cells (113). The transcription factor Krüppel-like factor 2 (KLF-2) promotes the expression of S1PR1, enabling tissue egress, but is downregulated after TCR activation. While IL-15 induces KLF-2, TNF-α, IL-33, and TGF-β cooperate to suppress its expression (128).

A central component of TRM differentiation is the modulation of T-box transcription factors, particularly T-bet and Eomesodermin (Eomes). Upon antigen encounter, activated $CD8^+$ T cells produce IL-2, which induces Eomes expression (129). Eomes is associated with the promotion of long-lived memory cells by enhancing self-renewal and proliferative potential (130). In contrast, T-bet is predominantly expressed in early effector $CD8^+$ T cells, where it supports cytotoxic function and inflammatory responses. As T cells transition to memory states, both T-bet and Eomes levels decline (131, 132). A low T-bet/Eomes expression ratio favors memory formation, and further downregulation of both factors is required for the acquisition of tissue residency. In the lungs, TRM cells expressing high levels of CD103 and CD69 exhibit notably suppressed T-bet levels (133). Similarly, in the skin, the downregulation of both Eomes and T-bet is critical for the infiltration, retention, and survival of TRM cells in the epidermis. Also, residual T-bet expression maintains CD122 expression and IL-15 responsiveness, supporting TRM survival in skin (89). Conversely, the expression of T-bet and Eomes is associated with CCR7, a receptor that promotes lymphoid tissue recirculation and favors the differentiation of $CD8^+$ T cells into TEM or TCM subsets (134). Thus, the coordinated repression of T-bet and Eomes can be one of the hallmarks of TRM differentiation, facilitating tissue adaptation and the quiescent phenotype necessary for long-term residency, particularly in epithelial barriers.

Runt-related transcription factor 3 (Runx3) is a transcription factor involved in cellular development, inflammatory responses,

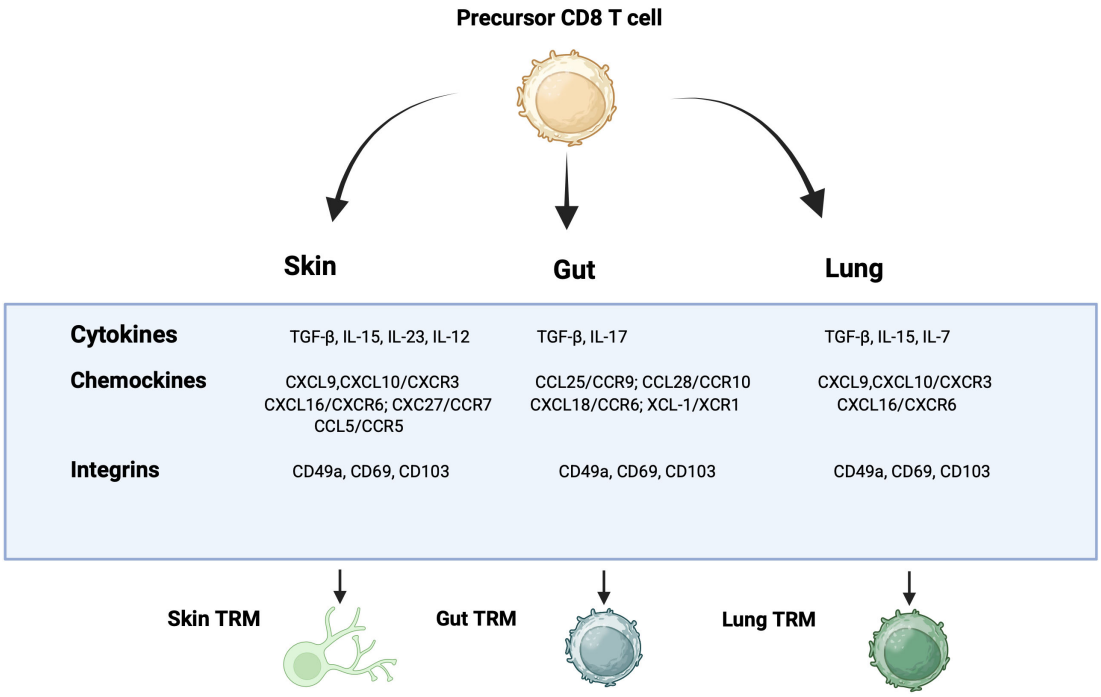



FIGURE 2
Cytokines, chemokines, and integrins involved in the differentiation of CD8 effector T cells into CD8 TRMs in the skin, gut, and lung.

and tumor suppression (135, 136). In the context of TRM biology, Runx3 has been identified as an essential regulator of lineage commitment and homeostasis (137). It promotes the expression of canonical TRM markers while repressing genes linked to tissue egress, including S1pr1 (128). Functional studies in murine models of LCMV infection and melanoma demonstrated that deletion of Runx3 results in a dramatic reduction, ranging from 50- to 150-fold in $CD8^+$ T cells expressing CD69 and CD103 across various tissues, including salivary glands, kidneys, skin, and lungs (138). Notably, Runx3 appears to function in a tissue-independent manner, reinforcing its role as a core component of the TRM transcriptional program. Runx3 regulates a transcriptional network that controls T cell migration and effector functions within non-lymphoid tissues (139). A deeper understanding of how Runx3 governs TRM differentiation may prove critical for advancing tissue-targeted immunotherapies that harness $CD8^+$ TRMs.

Additional transcription factors also contribute to TRM development in a tissue-specific manner. After effector $CD8^+$ T cells infiltrate tissues such as the skin, small intestine, liver, and kidneys, the expression of Hobit and Blimp-1 becomes essential for further differentiation. These factors promote TRM formation by repressing genes involved in circulation, such as CCR7 and S1PR1. Although Blimp-1 is not strictly essential, it cooperates with Hobit to drive the expression of TRM-associated surface markers including CD49a, CD69, and CD103 (126, 140). During pulmonary influenza infection in mice, Blimp-1 expression in terminal effector $CD8^+$ T cells favor the development of TRMs over TCMs. In this model, once effector $CD8^+$ T cells reach the lungs, Blimp-1 suppresses the expression of T cell factor-1 (TCF-1) in $CD8^+$ TRM cells, a mechanism that promotes TRM differentiation over TCM, as TCF-1 is essential for the development of the TCM lineage. This transcriptional program may facilitate the conversion of circulating $CD8^+$ T lymphocytes into tissue-resident cells, with Blimp-1 acting as a key regulator by antagonizing the TCM differentiation pathway (126). In the context of chronic viral infection, as shown in LCMV models, Hobit expression is confined to the TRM lineage, whereas Blimp-1 is broadly expressed across all $CD8^+$ effector and memory subsets (141). These findings suggest that Hobit is a more specific marker of TRM identity, while Blimp-1 serves a broader role in memory $CD8^+$ T cell differentiation by inhibiting TCF-1 and promoting TRM commitment.

Another key factor, the basic helix-loop-helix family member e40 (Bhlhe40), has recently gained attention for its role in sustaining $CD8^+$ TRM cell metabolism and function, particularly under inflammatory conditions. Initially studied in pulmonary TRMs, where it supports mitochondrial function and cytokine production (e.g., IFN-γ) (142). Bhlhe40 has also been shown to be essential for TRM maintenance in other tissues. In the brain, Bhlhe40-deficient $CD8^+$ T cells exhibit impaired persistence and effector function following viral infection. In the small intestine, Bhlhe40 limits T cell exhaustion and sustains TRM metabolic fitness during chronic infection (143). In the liver, it contributes to the regulation of intrahepatic TRM homeostasis and modulates inflammatory potential during hepatotropic viral infections (144). Moreover, Bhlhe40 expression is suppressed by PD-1 signaling, and its regulation has been implicated in the responsiveness of TRMs to anti-PD-1 immunotherapy (145). Thus, some transcription factors involved in TRM formation are common across most tissues, while others are more tissue-specific, as shown in Figure 3.

Altogether, these findings demonstrate that $CD8^+$ TRM cell fate is orchestrated by a coordinated transcriptional network. This includes repression of recirculation-related genes (e.g., S1pr1 and CCR7) and upregulation of residency and survival markers (e.g., CD69, CD103, CD49a). T-bet and Eomes downregulation permits adaptation to tissue niches; RUNX3 acts as a master regulator of the TRM program; Hobit and Blimp-1 further suppress TCM-related transcriptional programs; and Bhlhe40 supports metabolic fitness and long-term TRM persistence. These transcriptional checkpoints not only define TRM identity but also represent promising targets for therapeutic manipulation. Figure 4 presents an integrated schematic of the main signals characterized in the differentiation of CD8 effector cells into TRMs. The signals may vary depending on the tissue type.

## 4 Targeting signals to improve CD8 TRM responses in viral infections for immunotherapy and vaccine development

Understanding the molecular cues that govern TRM generation and maintenance has direct implications for the design of viral vaccines and antiviral immunotherapies. The induction of efficient TRMs can add an additional layer of peripheral protection, helping to contain the infection at barrier sites. Recent research into the activation of precursor cells and the generation of antiviral $CD8^+$ TRMs underscores the critical role of APC and MHC: TCR signaling in this process (47, 50, 53). Furthermore, in the context of Influenza A or Polyoma viral infections, $CD8^+$ TRMs are better generated in suboptimal TCR stimulation (60, 61). In addition, these Polyomavirus specific TRM cells exhibited higher-affinity TCRs compared to circulating memory T cells, and once established, antigen presence was no longer essential for TRM retention (59). Based on these observations, it can be inferred that antiviral $CD8^+$ TRMs generation was facilitated upon lower TCR stimulation, and the elevated TCR affinity of these cells enhances their ability to detect infected cells, including even those that expressed low levels of antigen. In vaccine or immunotherapeutic strategies aimed at expanding $CD8^+$ TRMs, approaches less dependent on antigen quantity may be advantageous. Instead, optimizing antigen delivery and release within the tissue microenvironment may more effectively promote tissue-specific immunity.

One promising approach to enhance $CD8^+$ TRMs in mucosal tissues involves the use of chemokines. In mouse models of genital HSV-2 infection, topical administration of CXCL9 and CXCL10 induced migration of $CXCR3^+$ $CD8^+$ T cells into the vaginal

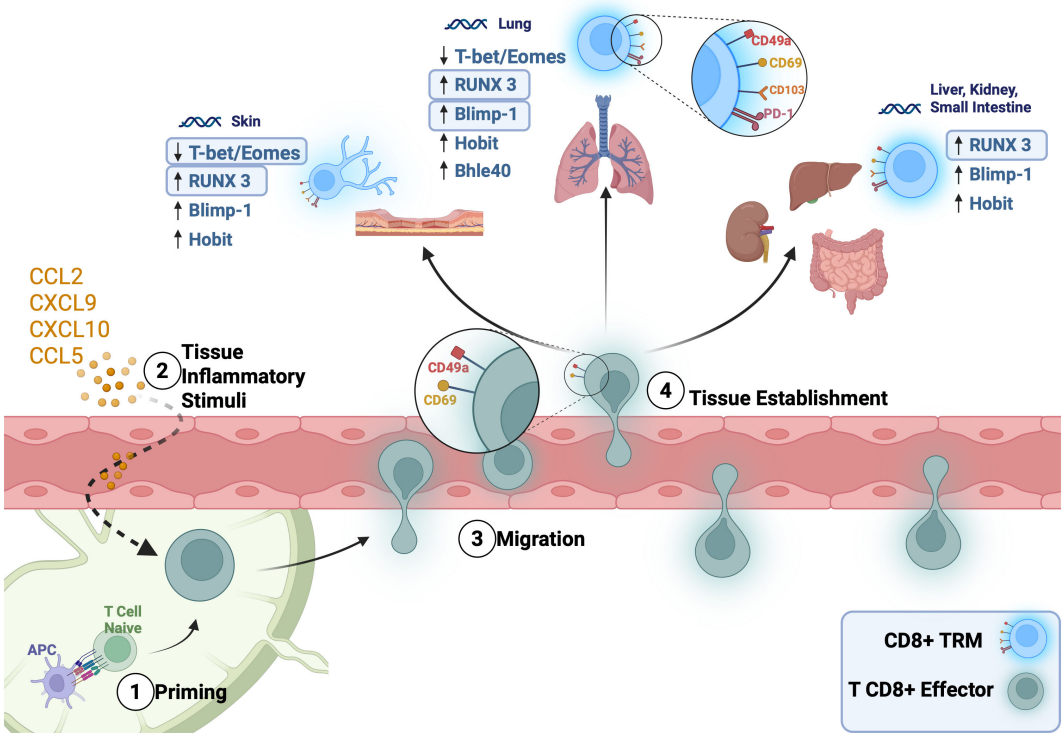



FIGURE 3
Migration and formation of CD8$^+$ TRMs in different tissues in the context of infection or injury and the expression of specific transcriptional factors. After being primed in the regional lymph nodes (1), and in response to inflammatory stimuli from the tissue that produces chemokines (2), effector CD8 T lymphocytes follow these signals and migrate into the inflamed tissue, expressing CD69 and CD49a (3). In this microenvironment, CD8+ T lymphocytes that express CD69 downregulate S1PR1, which leads to their retention in the tissue (4). Within each specific tissue, CD8 T lymphocytes respond to TGF-β and other local microenvironmental cues that induce transcription factors, promoting the expression of CD49a and other canonical functional markers of TRMs, such as CD103 and PD-1.

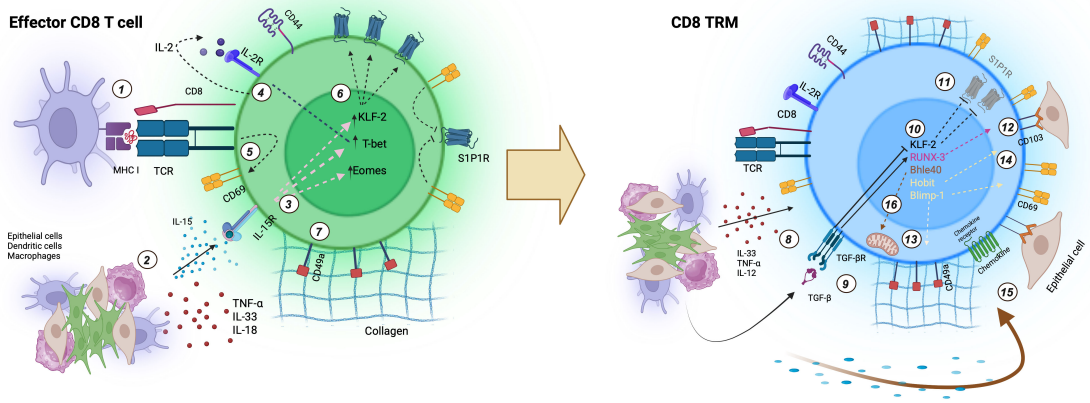



FIGURE 4
Integrated model of signals driving CD8$^+$ TRM differentiation: 1) TCR engagement with peptide–MHC I complexes (low or high affinity) provides activation signals; 2) Local cues derived from pathogens, injury, and inflammation—including TNF-α, IL-18, IL-33, and IL-15—further shape TRM differentiation; 3) IL-15 trans-presentation induces the transcription factors KLF2 and T-bet/Eomes; 4) Autocrine IL-2 reinforces T-bet expression; 5) TCR activation upregulates CD69 expression; 6) KLF2 promotes S1PR1 expression, regulating tissue egress; 7) Effector CD8$^+$ T cells begin expressing CD49a; 8) Production of TNF-α, IL-18, and IL-33 is maintained or amplified locally; 9) Epithelial cells, macrophages, and dendritic cells produce TGF-β, which binds to TGF-β receptors on CD8$^+$ T cells. 10) TGF-β signaling downregulates KLF2 and upregulates Runx3 expression; 11) Loss of KLF2 leads to reduced S1PR1 expression, favoring tissue retention; 12) Runx3 enhances CD103 expression; 13 and 14) Hobit and Blimp-1 cooperate with Runx3 to promote CD103, CD49a, and CD69 expression, consolidating tissue residency; 15) CD8$^+$ T cells express chemokine receptors that control tissue migration and morphology; 16) Sustained TCR, TNFα, TGF-β, and IL-33 signaling maintains Bhlhe40 expression, which supports metabolic homeostasis, oxidative stress resistance, and reinforces the TRM transcriptional program.

mucosa, where they differentiated into long-lived TRMs that contributed to viral control (146). This strategy could complement conventional vaccines that generate systemic T cell responses, potentially increasing the number of vaginal $CD8^+$ TRMs in various genital mucosal sexually transmitted infections. In genital HSV-2 infection, the response to infected cells is modulated by IFN-γ secreted by $CD8_+$ TRM lymphocytes (147).

Numerous vaccine models in mouse and immunotherapies for viruses have been extensively studied. These studies highlight the importance of considering key biological factors of lymphocytes—such as activation levels, cytokine responses, and their location within the body—when designing more effective vaccines and immunotherapies to enhance immune responses (148, 149). For example, with mRNA vaccines, the mRNA is engulfed by APCs, and recognized by endosomal and cytosolic innate sensors (150). This recognition triggers cellular activation and a strong generation and programming of antiviral T cells against the cognate antigen (151). The mRNA vaccine has been shown to effectively generate antibody and lymphocyte responses against respiratory pathogens in mice, rabbits and ferrets (152, 153). However, little is known about whether this vaccine model can also induce $CD8^+$ TRMs in lung tissue. In a murine model of mRNA vaccination against influenza, the formation of virus specific $CD8^+$ TRMs was observed in the lungs when the vaccine was administered either intramuscularly or intranasally. Additionally, prime-boost immunizations in mouse with intramuscular injection followed by intranasal mRNA vaccination led to high levels of both circulating and lung memory T cells against influenza (154). Indeed, while the initial dose can be administered systemically, optimal TRM responses require the booster dose to be delivered directly to the target tissue. This has been demonstrated in experimental mouse models involving genital (155) and lung tissues (156).

The most robust TRM responses are consistently achieved through tissue-targeted stimulation. For instance, immunization via the pulmonary route elicits stronger TRM responses in the lungs, and similar effects are observed when the skin or genital tract is used as the site of immunization. In the ongoing effort to improve vaccine responses to influenza, it was observed that intranasal immunization with S-FLU, a replication-deficient influenza virus vaccine (157), generates specific $CD8^+$ TRM lymphocytes in the lung, which are associated with protection. Interestingly, in this model, the characteristic of $CD8^+$ TRMs lymphocyte being preferentially generated from clones with lower TCR affinity enhances clonal diversity. This diversity in the receptor allows for the recognition of mutated peptide sequences, which can provide heterosubtypic responses of $CD8^+$ TRMs and reduce viral escape (158).

SARS-CoV-2 studies have provided further insights into TRM biology in humans. Individuals who were vaccinated or naturally infected developed nasal $CD8^+$ TRMs expressing CD49a that produced IFN-γ upon antigenic stimulation (159). Exploring $CD8^+$ TRM signaling might be a useful approach to improving SARS-CoV vaccines. In mice, intranasal vaccination with the viral receptor-binding domain (RBD) combined with polyethyleneimine, a potent mucosal adjuvante for viral glycoprotein antigens (160), demonstrated that this combination significantly expanded specific $CD4^+$ and $CD8^+$ TRM lymphocytes in lung tissue. These TRMs were bifunctional, producing both IFN-γ and TNF-α, with durability observed in lung tissue for up to one year following intranasal immunization (161). In humans, although the mRNA vaccine against SARS-CoV-2 induced TRM populations in lung tissue, wild type virus elicited a more robust response. The mRNA vaccine generated TRM cells $CD69^+CD103^+$ cells which produced only IFN-γ and failed to persist for very long time in the tissue. However, during infection, the TRMs phenotype were $CD69^+CD103^+$, produced IFN-γ, expressed CD107a, and exhibited a longer lifespan. An optimized lung TRM immune response in humans was observed when vaccination occurred after SARS-Cov 2 infection (162).

Immunization or immunotherapy strategies that target the signals responsible for generating and maintaining CD8 TRM lymphocytes should be prioritized for investigation and development. TRMs are strategically positioned for optimal protection, programmed to mount a rapid response, and can exhibit long-lasting persistence in tissues. These characteristics represent the gold standard for achieving a more effective and durable protective immune response. Perhaps we can deliver antigens to target the TRM response and combining cytokines or chemokines to activate, expand or recruit more cells into the tissue or even use transcription factors modulators in TRMs. However, what remains unknown is how and to what extent TRMs respond to the signals previously characterized for TCM and TEM cells, such as for expression of IL-2, IL-15, IL-21 and IL-7 receptors, CD4-help inside the tissue, cross-presentation and dependence of cytokine for differentiation. Further research is needed to understand how to tailor the TRM response to optimally protect against various pathogens.

## 5 The presence and functionality of TRMs in tumoral microenvironment: perspectives for immunotherapy improvement

More recently, TRMs have become the focus of several studies in tumor immunology. Historically, tumor-infiltrating lymphocytes (TILs) were associated with favorable prognosis. However, as molecular characterization of TILs advanced, it became evident that their differentiation state was a critical determinant of clinical outcomes. While memory T cells (163) were strongly associated with protection and good prognosis, T reg cells (164) and exhausted cells (165) are more associated with poor survival and unfavorable outcomes.

Among the protective memory TILs, $CD8^+CD103^+$ T cells have emerged as the subset most consistently associated with beneficial outcomes across diverse tumor types. In non–small cell lung cancer (NSCLC), increased intraepithelial lymphocyte infiltration and higher patient survival correlated with an expanded $CD103^+$ TIL population (166). In that study, freshly isolated $CD8^+CD103^+$ TILs from NSCLC specimens displayed transcriptomic and phenotypic

profiles characteristic of TRMs. Similar associations with favorable prognosis were subsequently observed in other NSCLC studies (167, 168), and also verified in human ovarian tumors (169) as well as in human breast tumors (170). Beyond its role in maintaining TRMs within tissues, CD103 is essential for the efficient adhesion of activated T cells to cancer cells. CD103-expressing T cells can lyse tumor cells expressing its ligand, E-cadherin (171). Interestingly, $CD8^+CD103^+$ TRMs often accumulate within fibrotic or stromal regions distant from the tumor epithelium, as seen in pancreatic cancer, suggesting that fibrosis and stromal architecture may physically restrict TRM access, functioning as a tumor immune-evasion mechanism (172).

In most, if not all, of the above-cited studies in humans, protective TRMs frequently express PD-1 and Tim-3 checkpoint receptors. Early single-cell transcriptomic analyses categorized TILs with this signature as exhausted (173–175). More recently, it has become clear that $PD\text{-}1^+$ TRMs represent the subset most closely associated not only with tumor protection but also with responsiveness to PD-1/PD-L1 checkpoint blockade in humans (174). In murine models, this TIL subset exhibits enhanced activation-induced cell death and mediates potent cytolytic activity toward autologous tumor cells upon blockade of PD-1–PD-L1 interaction (176).

## 5.1 Signals from tumoral microenvironment to TRMs

Similar to virus-infected tissues, $CD8^+$ TRMs in tumors must integrate multiple environmental cues to sustain their persistence and antitumor function. Among these, antigen recognition plays a central role in driving TRM activation and differentiation (177). TCR repertoire analyses in human colorectal and gastric cancers show that TIL clonotypes are oligoclonal and enriched for tumor-associated antigens (178, 179). However, many early studies did not distinguish TRMs from other TIL subsets. More recent deep-sequencing data demonstrate that TRM-enriched lesions in melanoma, glioblastoma, and head and neck cancers display distinct TCR diversity shaped by the local antigenic landscape (180–182). Transcription factors governing tissue residency and long-term immune surveillance regulate gene programs that sustain TRM persistence and effector capacity within tumors (183). Additionally, the metabolic milieu of the tumor microenvironment critically influences TRM activity, depending on nutrient availability and hypoxic stress (178). In this section, we will discuss these key factors, highlighting their collective impact on the biology of intratumoral TRMs and their role in shaping the antitumor immune response.

Intratumoral effector $CD8^+$ T cells receive antigen-driven signals through TCR stimulation, which promotes their differentiation into $CD8^+$ TRMs. Initially, these findings were described in human pancreatic tumors (171). Subsequently, it was demonstrated that the expression of CD103/β7 in human colon carcinoma-specific CTLs is synergistically enhanced by the simultaneous stimulation of TGF-β1 and antigen recognition (179). Antigen dependency of $CD8^+$ TRM function was also confirmed in murine brain tumor models and human malignant glioma samples. Functionally, these cells were restimulated as effectors and release IFN-γ and granzyme B (184). Additionally, highly expanded T cell clones tend to accumulate within the tumor microenvironment, leading to a more restricted and oligoclonal TCR repertoire compared to peripheral T cells (182). However, these studies categorize TRMs as part of the broader TIL population without specifically distinguishing them as a unique subset. This lack of differentiation limits our understanding of the specific contributions of TRMs to tumor immunity.

On the other hand, metastatic melanoma lesions in human were observed to be enriched with TRMs, suggesting their involvement in localized immune responses within tumor sites. Notably, these lesions exhibit intralesional TCR diversity, as revealed by deep sequencing of sorted cells for the TCRβ locus. This diversity is likely driven by differences in tumor mutations or neoepitope landscapes among metastases (180). Similar findings also were reported in other malignancies, including human head and neck cancers (185), mouse glioblastoma (186), human vaginal melanoma (187), human oral cavity squamous cell carcinoma (188), and human multiple primary lung cancers (189). Additionally, TRM clonotypes originally identified in tumors were detected in the skin and blood of metastatic melanoma survivors for up to nine years post-treatment (190). Collectively, these studies reinforce the concept that TRMs are key orchestrators of local antitumor immunity and exhibit remarkable adaptability to distinct tumor antigenic environments.

In addition to the TCR in antigen recognition by TRMs, the CD103 molecule plays a fundamental role in enabling these cells to combat tumor growth effectively. Accordingly, CD103 is recruited to the immunological synapse, and its interaction with E-cadherin is essential for cytolytic granule polarization and subsequent exocytosis, leading to efficient tumor cell lysis by tumor TILs. Mechanistically, CD103 ligation triggers phosphorylation of PLCγ1 and ERK1/2, which orchestrate actin remodeling and directed secretion of granzyme and perforin. Inhibiting PLCγ blocks granule relocalization, thereby reducing TCR-mediated cytotoxicity (191). Notably, the interaction between αEβ7 on TILs and E-cadherin on autologous CCL3-producing tumor cells facilitates CCR5 recruitment at the immunological synapse. Moreover, the chemokines CCL3 and CCL5 selectively drive the accumulation of $CD103^+$ $CD8^+$ T cells, a mechanism observed in human NSCLC (192). Notably, CD103 expression is induced on $CD8^+$ T cells following TCR engagement and exposure to TGF-β1, with the transcription factors Smad2/3 and nuclear factor of activated T cells 1 (NFAT-1) serving as critical regulators of this process (193).

Beyond CD103, the CXCR6–CXCL16 axis is emerging as a key determinant of TRM retention, positioning, and survival in tumors (194, 195). CXCR6 deficiency leads to defective TRM recruitment, impaired vaccine responses, and reduced survival in mouse models of head and neck and lung tumors (196). n a mouse pancreatic cancer model, transduction of CXCR6 into CAR-T cells targeting mesothelin (CART-MSLN) promoted tumor rejection and durable

remission (197). CXCR6 expression correlates with effector transcriptional programs underscoring the antitumor activity of TRMs in mice (198). Conversely, CXCR6-deficient T cells display increased apoptosis, with gene-set enrichment analyses revealing downregulation of the CD28 signaling pathway, further confirmed by flow cytometry (199). CXCL16, the ligand of CXCR6, plays a pivotal role in T-cell retention and is primarily expressed by antigen-presenting cells (200). However, tumor cells can also express CXCL16 (201) and high levels of this chemokine in tumor cells correlated with increased T-cell infiltration and prolonged patient survival in colorectal cancer (202). Importantly, engineering CAR-T cells to express CXCR6 or RUNX3 enhances their intratumoral persistence and tumor control, highlighting the translational potential of modulating this pathway (203, 204).

Cytokines also serve as critical signaling factors for TRMs. In bladder cancer, TRMs can be activated by anti-CD3, anti-CD28, and IL-15 stimulation, leading to proliferation and increased T-bet, perforin, and granzyme B expression, particularly in the PD-$1^+$ subset (205). The importance of IL-15 in cancer-associated TRMs has also been demonstrated in melanoma, where intratumoral IL-15 expression varies among patients and shows a positive correlation with TRM abundance. Furthermore, IL-15 expression is strongly associated with increased mRNA levels of $CD8^+$ TRM-related markers, including ITGAE (CD103), ITGA1 (CD49a), and CD69, reinforcing its role in shaping the intratumoral T cell landscape (206). IL-15 plays a central role in sustaining TRM function and cytotoxicity within tumors. TRMs isolated from bladder cancer patients proliferate and upregulate T-bet, perforin, and granzyme B in response to IL-15 stimulation (205). In melanoma, IL-15 expression positively correlates with TRM abundance and higher mRNA levels of ITGAE (CD103), ITGA1 (CD49a), and CD69, reinforcing its role in maintaining residency and effector potential (206). These findings suggest that IL-15 plays a crucial role in sustaining TRM function and cytotoxic potential within the tumor microenvironment.

## 5.2 Perspectives for advanced TRM based immunotherapies for cancer

Recently, several immunotherapeutic strategies and clinical trials targeting $CD8^+$ TRMs have been explored. One such approach is Chimeric Antigen Receptor T-cell (CAR-T) therapy, a personalized treatment in which patient-derived lymphocytes are isolated, genetically modified to express a chimeric antigen receptor (CAR), and then re-infused into the patient to enhance tumor cytotoxicity (207). A preclinical study using a liver cancer model demonstrated that overexpression of Runx3 in CAR-T cells significantly improved both the persistence and the infiltration of T cells within tumor tissues. This modification not only enhanced the antitumor activity of CAR-T cells but also contributed to prolonged therapeutic efficacy, offering potential for sustained response in solid tumors (208).

Radiotherapy remains a critical modality in the treatment of solid tumors. While T lymphocytes are radiosensitive, a study in a murine model of colon adenocarcinoma revealed that pre-existing intratumoral T lymphocytes can survive radiotherapy. Notably, the tumor microenvironment appears to reprogram these lymphocytes, inducing a molecular signature similar to that of TRMs. This includes increased expression of CD49a and E-cadherin, along with a reduction in S1P1R expression. These findings suggest the potential for combining radiotherapy with immunotherapy strategies targeting TRMs to enhance therapeutic outcomes (209).

Vaccination models and strategies designed to induce the generation of $CD8^+$ TRM lymphocytes specific to tumor antigens are actively being investigated and have demonstrated promising results *in vivo*. For instance, breast cancer, a leading cause of cancer-related mortality worldwide, has a propensity to metastasize the lungs. A nasal mucosa vaccine model utilizing an adenoviral vector encoding tumor-associated antigens demonstrated that both prophylactic and therapeutic applications significantly increased the number of $CD8^+$ TRMs in lung tissue. This increase was directly correlated with enhanced control of pulmonary breast cancer metastasis in the prophylactic setting. Furthermore, when combined with radiotherapy, the vaccine synergistically enhanced the relationship between TRMs and therapeutic efficacy, highlighting the potential for combining vaccination and radiotherapy as an integrated approach to cancer treatment (210). Similarly, systemic prime-intranasal boost immunization also provided protection against breast cancer metastasis to the lung. In this model, $CD8^+$ TRMs exhibited polyfunctional capacity, secreting key cytokines such as IFN-γ, IL-2, and TNF-α, further underscoring their pivotal role in antitumor immunity (156). Vaccine models of TRM induction also were effective in cutaneous melanoma (211) and HPV-induced carcinomas in the skin and liver (212).

Mechanistic dissection of these pathways has prompted efforts to develop a standardized "TRM immunoscore" that integrates phenotypic composition (CD103, CD69, CD49a) with spatial localization relative to tumor epithelium and vasculature. This framework may help distinguish prognostic biomarkers (linked to survival) from predictive biomarkers (linked to therapeutic response) (213, 214). However, technical limitations remain, such as marker loss during enzymatic dissociation and variable sample processing, which may underestimate TRM frequencies. Standardized protocols and spatially resolved transcriptomic tools will be essential for accurate TRM profiling. Altogether, tumor-resident $CD8^+$ TRMs act as dynamic epithelial sentinels that integrate antigenic, adhesive, and cytokine cues to maintain residency and sustain cytotoxic competence, representing a critical target for next-generation immunotherapies.

# 6 Conclusion

This review highlights the pivotal role of TRMs in both immunity to infections and cancer immunotherapy. It emphasizes the unique differentiation and functional properties of TRMs. These cells are formed from differentiated effector $CD8^+$ T cells and possess the distinct advantage of residing permanently in tissues.

This enables them to provide rapid, localized responses upon re-infection or tumor detection. Their formation is influenced by a complex interplay of antigen recognition, tissue-specific cytokine signals, and transcriptional regulation, with key markers such as CD69, CD103, and CD49a ensuring their retention and function in tissues.

The comparison between viral infections and cancer environments reveals both commonalities and challenges. While the mechanisms of TRM formation share similarities across these contexts, cancer microenvironments present additional immunosuppressive signals that complicate the maintenance of effective TRM responses. The presence of checkpoint receptors such as PD-1 and Tim-3 on TRMs in tumors is indicative of the complex balance between protection and immune evasion. This points to the potential benefits of combining TRM-targeted immunotherapies with immune checkpoint blockade.

The use of $CD8^+$ TRM lymphocytes as therapies for infections and cancer faces several challenges. The effective generation of these cells depends on local stimuli, whereas most immunization and immunotherapy strategies still rely on systemic routes, such as intramuscular or intravenous administration. Another obstacle is the limited understanding of the plasticity among memory T cell subpopulations, particularly regarding the potential conversion between TCMs and TRMs, a critical aspect for the regulation of immunological memory. Moreover, much of the current knowledge is derived from murine models, while human studies remain in pre-clinical stages. The difficulty in isolating and manipulating TRMs ex vivo also poses a significant technical limitation. The expression of the PD-1 marker, involved in the functional regulation of TRM cells, remains under investigation in various contexts, particularly regarding its role in the response of these cells to TGF-β (215). Finally, the local activation of these cells may trigger exacerbated inflammatory responses, raising safety concerns. The formation, maintenance, and function of TRMs still depend on molecular and environmental signals that are not yet fully understood, limiting their safe and effective clinical application.

In summary, the regulation of $CD8^+$ TRM lymphocytes is a cornerstone of immune responses, providing long-term protection against infections and playing a crucial role in cancer immunotherapy and vaccine optimization. The emerging strategies that aim to enhance TRM function, through either vaccine optimization or immunotherapy, hold great promise for improving the durability and specificity of immune responses, contributing to more effective treatments for both infectious diseases and cancer. Further research into TRM biology is essential for refining these therapeutic strategies and realizing their full potential in clinical settings.

## Author contributions

LR: Funding acquisition, Writing – original draft, Formal analysis, Writing – review & editing, Investigation, Validation, Supervision, Visualization, Software, Data curation, Project administration, Conceptualization, Resources, Methodology. CB: Writing – original draft, Writing – review & editing. AH: Writing – review & editing. ML: Writing – original draft, Visualization, Conceptualization. GP: Visualization, Writing – original draft. PR: Writing – review & editing, Visualization. BR: Investigation, Writing – review & editing, Validation, Supervision, Formal analysis, Software, Visualization, Data curation, Project administration, Funding acquisition, Conceptualization, Resources, Writing – original draft, Methodology.

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## Conflict of interest

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# 8. ANEXOS

## 8.1. Parecer do Comitê de Ética no uso de animais da UFCSPA

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Pró-Reitoria de Pesquisa e Pós-Graduação

Comissão de Ética no Uso de Animais - CEUA

# CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais analisou o projeto:

Número: **924/2024**
Título: **Estudo da dinâmica de linfócitos T residentes de memória de mucosa genital na infecção por Herpes Simplex Virus-2 e influência da terapia com Aciclovir**
Pesquisador(a) Responsável: **Luiz Carlos Rodrigues Junior**
Vigência: **15/03/2024** a **25/02/2026**

Pesquisadores:

Equipe UFCSPA:

- Luiz Carlos Rodrigues Junior
- Alisson Felipe Haubert
- Helena Dörr Toebe
- Karla Maria Del Passo

Equipe Externa: Não possui.

*A Comissão de Ética no Uso de Animais aprovou o mesmo em reunião.*

**Parecer:**

| Animal | Espécie | Linhagem | Origem | Idade | Peso aprox. | Quantidade | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | M | F | Subtotal |
| Camundongo | isogênico | - | biotério a ufcspa | 2 mês/meses | 20 gramas | 0 | 168 | 168 |
| | | | | | | Total | | 168 |

Porto Alegre, 19 de setembro de 2024

FERNANDA BASTOS DE MELLO

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE CIÊNCIAS DA SAÚDE DE PORTO ALEGRE

Coordenadora Da Comissão De Ética No Uso De Animais

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