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Eduardo Cheuiche Antonio

Investigando os aspectos estruturais que conferem imunogenicidade diferencial em epítopos de células T tumorais

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel em Biomedicina.

Orientador: Dr. Gustavo Fioravanti Vieira

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Dedicatória

Gostaria de agradecer a todos os meus amigos, família e colegas de trabalho por me acompanharem em uma longa estrada. O apoio de todos foi o alicerce principal que me permitiu chegar aqui e poder compartilhar este trabalho com a comunidade cientifica e o resto do mundo. Espero que fiquem orgulhosos.

Everything has its wonders, even darkness and silence, and I learn, whatever state I may be in, therein to be content.

Helen Keller.

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RESUMO

A busca por quais características definem um epítopo como um alvo imunogênico ou não responsivo para imunoterapia tem iludido pesquisadores há anos. Vários estudos demonstram que certas posições nas sequências peptídicas, os resíduos âncora do MHC, têm uma composição preferencial de aminoácidos (motivos alélicos), sendo esses epítopos mais susceptíveis de exibir uma melhor resposta imunogênica. Em primeiro lugar, nem todos os ligantes de MHC são imunogênicos, considerando que temos epítopos não numerados sendo continuamente apresentados nas superfícies das células. Neste trabalho específico, testamos um elemento adicional, central em nossa hipótese de que alterações nas sequências de proteínas tumorais resultam em uma mudança estrutural que desloca a superfície eletrostática das moléculas pMHC, fundamental para o reconhecimento de TCR e o início de uma resposta imunogênica. Para este fim, foram recuperadas sequências de neoepitopos apresentando respostas imunes diferenciais quando comparadas com suas contrapartes do tipo selvagem. Apesar do fato de as sequências serem muito semelhantes, elas desencadearam respostas que eram consideravelmente diferentes e, atualmente, não há uma explicação bem estabelecida de por que elas diferem visivelmente nos aspectos imunogênicos entre si. Estruturas pMHCs abrigando as sequências do epítopo foram modeladas e usadas para gerar imagens de suas superfícies eletrostáticas, procurando diferenças qualitativas que possam indicar as respostas distintas. Observamos que nenhuma alteração significante ocorreu entre os peptídeos tumorais imunogênicos e suas contrapartes não-imunogênicas de tipo selvagem quando comparamos sua superfície eletrostática. Uma comparação adicional foi feita contra estruturas de pMHCs contendo epítopos imunogênicos recuperados da Crosstope Database (www.crosstope.com). Nesse sentido, também foi possível verificar se os epítopos tumorais imunogênicos eram semelhantes aos imunogênicos virais. Surpreendentemente, tanto as sequências não tumorais quanto os neoepitopos compartilharam uma similaridade na distribuição de superfície eletrostática com os alvos patogênicos, o que poderia ser um indicativo de sua predisposição imunogênica. Portanto, teorizamos que um "elemento oculto" pode ser responsável pela mudança na imunogenicidade dos neoepitopos.

Palavras-chave: Neoepitopos. Estrutura de complexos pMHCs. Padrões imunogênicos.

ABSTRACT

The search for what characteristics define an epitope as either an immunogenic or a non-responsive target for immunotherapy has eluded researchers for years. Several studies demonstrate that certain positions in the peptide sequences, the MHC anchor residues, have a preferential composition of amino acids (allelic motifs), being those epitopes more likely to display a better immunogenic response. First of all, not all MHC ligands are immunogenic, considering that we have unnumbered self-epitopes being continuously presented in the cell surfaces. In this specific work, we tested an additional element, central in our hypothesis that alterations in tumor protein sequences result in a structural change that shifts the electrostatic surface of the pMHC molecules, pivotal for TCR recognition and the initiation of an immunogenic response. Then, previously neoepitope sequences presenting differential immune responses when compared with their wild-type counterpart were recovered. Despite the fact that the sequences were very similar, they triggered responses that were considerably different, and currently, there is no well-established explanation of why they conspicuously differ in immunogenic aspects to each other. pMHCs structures harboring the epitope sequences were modeled and then used to generate images of their electrostatic surfaces, looking for qualitative differences that can indicate the distinct responses. We noticed that no significant alteration occurred between immunogenic tumor peptides and their wild-type non-immunogenic counterparts when comparing their electrostatic surface. An additional comparison was made against structures of pMHCs containing immunogenic epitopes recovered from the Crosstope Database (www.crosstope.com). In this sense, it was also possible to verify if immunogenic tumor epitopes were similar to viral immunogenic ones. Surprisingly, both WT sequences and neoepitopes shared an electrostatic surface distribution with pathogen targets, which could be an indicative of their immunogenic predisposition. So we theorized that a “hidden element”, may be responsible for the immunogenicity shift in neoepitopes.

Keywords: Neoepitopes. Structure of pMHCs complexes. Immunogenic Patterns.

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1 INTRODUÇÃO COMPREENSIVA

1.1 O SISTEMA IMUNE E A RESPOSTA CITOTÓXICA

O Sistema Imunológico identifica seus alvos através do reconhecimento de assinaturas moleculares apresentadas por um patógeno específico (JANEWAY; MEDZHITOV, 2002). O processo de reconhecimento identifica padrões moleculares que estão presentes em patógenos / micróbios, mas não encontrados nas células do hospedeiro (MEDZHITOV; JANEWAY, 2000). O reconhecimento imunológico inato depende de receptores com ampla especificidade e pode potencialmente se ligar a um grande número de moléculas que compartilham uma estrutura semelhante ou apresentam o mesmo padrão molecular (MEDZHITOV, 2007).

A resposta imune adaptativa, similar ao sistema inato, funciona através de mecanismos de reconhecimento de próprio e não próprio; tal identificação feita pelo sistema adaptativo é baseada na via de apresentação de antígenos (HOUGHTON; GUEVARA-PATIÑO, 2004). Nossas células que não estão infectadas ou mutadas apresentam auto antígenos que são diferentes daqueles na superfície das bactérias ou aqueles encontrados em células hospedeiras infectadas por vírus que classificam então como não-próprios (GOLDMAN; PRABHAKAR, 1996). A resposta adaptativa pode ser dividida em duas respostas principais que são coordenadas por duas células diferentes: a resposta celular, que é orquestrada pelas células T $CD8^+$; e a resposta humoral, que é coordenada pelas células B. (JANEWAY JR et al., 2001).

Os linfócitos T CD8 são as células responsáveis pela eliminação de patógenos intracelulares, como vírus e células anormais, como as células cancerígenas. Elas carregam em sua superfície os receptores de células T (TCR, do inglês *T-cell receptor*), moléculas capazes de reconhecer os fragmentos de antígenos associados a molécula de MHC (do inglês, *major histocompatibility complex*). Através da interação da molécula de MHC associada a um peptídeo (pMHC) e do TCR, eles são capazes de reconhecer partículas virais apresentadas pela molécula de superfície do MHC I no caso de uma infecção viral e também capazes de detectar proteínas mutadas geralmente exibidas no contexto de câncer. Os peptídeos gerados no citosol pela maquinaria celular são transportados para o retículo endoplasmático pelo Transportador associado ao Processamento de Antígenos (TAP, em inglês), onde se associa a outras proteínas para formar o Complexo de Carregamento de Peptídeos. Neste complexo, a montagem e o carregamento dos MHCs do tipo I ocorre com o auxílio das proteínas Tapasinas e culmina na inserção do peptídeo na fenda. A partir do evento de encaixe, a

afinidade entre peptídeo e MHC dita a conclusão do processo: Se a afinidade for baixa, o peptídeo escapará da fenda e o MHC entrará em uma rota de “desmonte”; e caso a afinidade seja alta o suficiente, o complexo irá emergir para superfície celular e será apresentado (BLUM; WEARSCH; CRESSWELL, 2013; SEWELL, 2012).

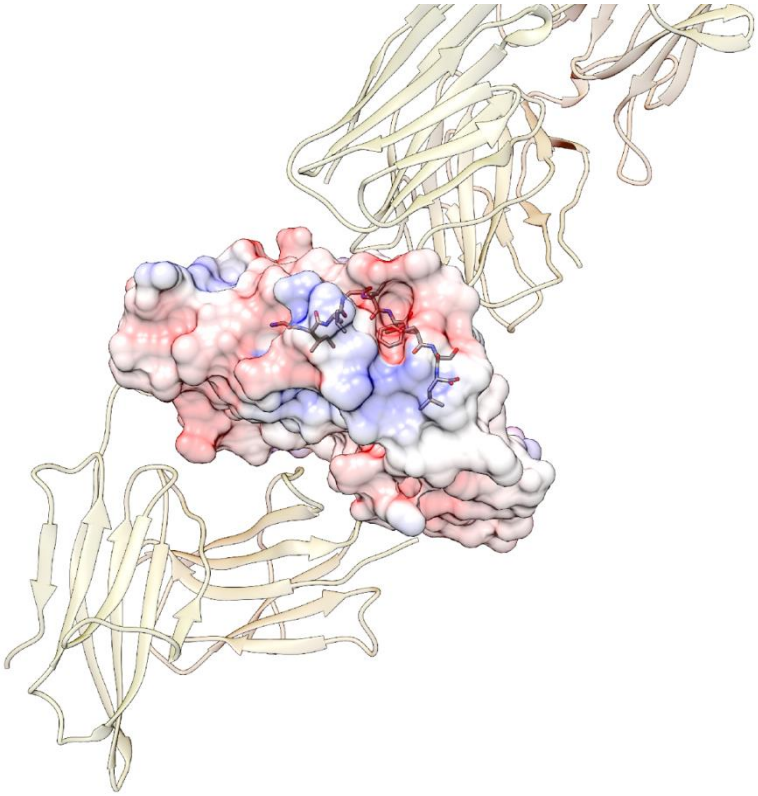

Figura 1. Uma representação esquemática de uma sinapse imunológica entre um MHC carregando um peptídeo e um TCR. Imagem gerada através do Software UCSF Chimera. Fonte: elaborado pelo autor.

1.2 CÂNCER: UMA BREVE REVISÃO

O corpo humano é composto de trilhões de células, e todas elas precisam de um controle orquestral de como elas se dividem, crescem e morrem para que não perturbem outras células em suas proximidades. O Câncer começa quando um grupo de células começa a crescer fora de controle e, por vezes, prolifera para outros tecidos do corpo. Em vez de morrerem, elas continuam a se espalhar e ao longo do tempo começam a criar novos tipos aberrantes de células, cada uma mais capaz de se replicar e sobreviver a duras condições até que elas se tornem capazes de viajar para outras partes do corpo e, uma vez lá, elas tenham a capacidade

de formar novos tumores; este processo é chamado de metástase e ele acontece quando as células do tumor primário caem na corrente sanguínea ou nos vasos linfáticos do corpo (AMERICAN CANCER SOCIETY, 2015). Existem vários processos celulares que um grupo de células precisa sofrer para poderem se tornar um tumor maligno: elas precisam sustentar sinais de proliferação ao mesmo tempo em que atingem a imortalidade replicativa para poderem manter o seu processo de mitose e crescer indefinidamente; é necessário parar os mecanismos supressores de crescimento da célula e resistir aos fatores intrínsecos e extrínsecos que desencadeiam a apoptose, a fim de sobreviver às tentativas do corpo de controlar o crescimento anormal que ocorre em tumores; é necessário ativar o processo de angiogênese, para que o câncer possa extrair mais oxigênio e nutrientes e para providenciar uma nova forma de invadir outros órgãos as células malignas, e, finalmente, o câncer tem que produzir células capazes de suportar todo o processo de metástase, o que inclui sobreviver a viagens no interior dos vasos sanguíneos ou vasos linfáticos e uma vez que cheguem a outro órgão, as células cancerosas precisam ser capazes de crescer no novo tecido [10] (HANAHAN; WEINBERG, 2011).

Uma das características mais proeminentes no câncer é a elevada taxa de mutação que geralmente confere às células neoplásicas a capacidade de se adaptar rapidamente a várias condições, como hipóxia e falta de nutrientes, e também confere à célula as ferramentas necessárias para manter a replicação constante e eventualmente conferir os meios para se espalhar para outras regiões do corpo em um processo conhecido como metástase (HANAHAN; WEINBERG, 2011; PAVLOVA; THOMPSON, 2016). O processo para adquirir novas características críticas para a sobrevivência das células cancerígenas, por vezes, leva à formação de proteínas mutantes chamadas Neoantigenos (AURISICCHIO *et al*, 2018).

Neoantígenos, ou Antígenos Tumorais Específicos (TSA, do inglês, *tumor-specific antigen*), são proteínas que não são expressas em células normais e são os resultados de mutações em sequências codificadoras de proteínas. A formação de neoantígenos pode ocorrer durante a própria transformação neoplásica ou devido ao aumento da instabilidade genética em locais não-críticos no genoma (na forma de mutações passageiro). O desenvolvimento de terapias que poderiam destruir os tumores com precisão sem prejudicar os tecidos saudáveis, como a quimioterapia clássica, tornou-se um dos maiores desafios da oncologia. Atualmente, a imunoterapia tem sido uma das principais candidatas para o papel de uma terapia capaz de eliminar as células cancerígenas com precisão, evitando as células

saudáveis. Embora imperfeita, a imunoterapia está mostrando grandes promessas, com o maior desafio para ela é identificar os alvos ideais para ela. Neste contexto, a vantagem da utilização de neoantígenos como alvos para imunoterapia é que os epítopos gerados pelas proteínas mutadas não foram submetidos à seleção tímica e tolerância central, portanto, o organismo deve reconhecer essas sequências como estranhas e desenvolver uma resposta imunogênica contra elas (WIRTH; KÜHNEL, 2017; KAKIMI *et al.*, 2017; ILYAS; YANG, 2015).

1.3 CÂNCER X SISTEMA IMUNE: O RACIONAL POR TRÁS DA IMUNOTERAPIA

No desenvolvimento neoplásico, quando o sistema imune detecta a anomalia e inicia uma resposta, ela geralmente se divide em três etapas: eliminação, equilíbrio e escape. Em um primeiro momento, o sistema imune consegue de maneira efetiva eliminar as células aberrantes e muitos começos de crescimentos aberrantes geralmente terminam nesta etapa. Caso células neoplásicas escapem deste processo, a etapa do equilíbrio começa, que é caracterizada pelo sistema imunológico manter as células neoplásicas em um estado de quiescência ou dormência funcional. Elas se mantêm neste estado devido à constante pressão imunológica e permanecem assim por períodos bastante variados, e em alguns casos, evoluem variantes celulares capazes de escapar do controle imunológico, caracterizando a fase de escape. Através de diversos mecanismos como perda das moléculas de MHC I na superfície, aumento de sinais de sobrevivência celular, desenvolvimento de um microambiente tumoral imunossupressor e outros mecanismos, o crescimento tumoral pode escapar do controle do sistema imune e começar a formar uma massa tumoral maligna (MITTAL *et al.*, 2014; DUNN; OLD; SCHREIBER, 2004). Através de uma análise racional por trás das três fases que o processo neoplásico sofre até culminar em uma massa tumoral, se torna evidente a necessidade de arquitetar formas de reativar ou potencializar a resposta citotóxica.

Partindo-se do pressuposto que o repertório de antígenos tumorais é composto em parte por proteínas próprias que sofreram mutação e de uma forma são então reconhecidas como imunogênicas pelo sistema imune, levantou-se a hipótese que talvez fosse possível o desenvolvimento de vacinas contra antígenos normalmente expressos por um determinado tipo de câncer (OVERWIJK *et al.*, 2003; ROSENBERG; YANG; RESTIFO, 2004). Outra abordagem além do desenvolvimento de vacinas para o tratamento de neoplasias foi o chamado de terapia celular adotiva (ACT, do inglês "*adoptive cell therapy*"), uma terapia personalizada que é baseada na expansão de linfócitos T *ex vivo* (sendo a utilização de células

T autólogas a mais eficiente) com a adição de moléculas que auxiliam na expansão celular como o IL-2 e sua posterior readministração em pacientes; regressões completas e duradouras em pacientes com melanoma já foram reportadas com essa técnica. O sucesso da ACT expandiu os horizontes das terapias celulares, levando ao desenvolvimento das pesquisas que envolvem a manipulação genética de linfócitos para que expressam receptores de células T quiméricos (ROSENBERG; RESTIFO, 2015; BLANKENSTEIN *et al.*, 2015; YEE *et al.*, 2002).

No campo de terapias para tumores malignos, a engenharia de células com receptores quiméricos vem sendo uma das técnicas mais proeminentes e com resultados bastante satisfatórios. As células T com receptor quimérico (CAR T-cell, do inglês, *Chimeric Antigen Receptor T-Cell*) possuem o seu receptor original substituído por um constructo contendo a região da cadeia variável de um anticorpo fundido com as cadeias de sinalização do TCR, assim, estas células possuem a capacidade de reconhecer diversos antígenos de maneira não restrita somente pelo peptídeo contido na fenda da molécula de MHC, com a vantagem de preservar toda atividade citotóxica de um linfócito T normal (ONG *et al.*, 2017). Os estudos em células CAR T já gerou quatro gerações distintas deste tipo celular quimérico, cada uma portando modificações em suas vias de sinalização. As CAR T de primeira geração possuíam o design mais simples, com um anticorpo de cadeia única CD3-ζ ou FcεRIγ unidos ao motivo de ativação baseado em tirosina da célula T ou ITAM (do inglês, *Immunoreceptor Tyrosine-based Activation Motif*). Um defeito das CAR T de primeira geração é que elas não produzem interleucina-2 (IL-2) suficiente para eliminar as células tumorais sendo necessário a coadministração de IL-2 de maneira exógena para garantir uma atividade citotóxica mais efetiva. As de segunda geração foram as primeiras a introduzir a adição de moléculas co-estimulatórias na via de sinalização das CAR T, sendo CD28 o receptor co-estimulatório mais utilizado na construção das CAR T de segunda geração. Este receptor aumenta a proliferação das células T quiméricas, somado com um aumento na expressão de citocinas, principalmente da molécula de IL-2 que garante a ativação da célula T e a promoção da sua atividade citotóxica. As CAR T de terceira geração adicionaram ainda mais domínios co-estimulatórios no processo de transdução de sinal, como por exemplo CD3ζ-CD28-OX40 ou CD3ζ-CD28-41BB. Tais combinações de moléculas co-estimulatórias se provaram eficientes para um aumento da liberação de IL-2 e um aumento da atividade de NFκB, que possui uma importante atividade na resposta imune. As CAR T de quarta geração foram gerados pela

adição de IL-12 na estrutura base da CAR T de segunda geração, tal adição aumenta a efetividade células T quiméricas que, em cascata, recrutam e estimulam outras células da imunidade inata para eliminar as células cancerígenas localizadas na região (ZHANG et al., 2017, WILKINS; KEELER; FLOTTE, 2017).

1.4 O ASPECTO ESTRUTURAL DE NEOANTÍGENOS NO CONTEXTO DE HLAS DO TIPO I

Vários estudos demonstram que proteínas mutadas no contexto do câncer podem exibir sequências de epítopos capazes de desencadear uma resposta imune. Considerando a grande eficiência das imunoterapias, a identificação de novos alvos para terapias de precisão utilizando células T se tornou fundamental para o desenvolvimento e aplicação efetiva de tais tratamentos, localizando os melhores alvos através de técnicas como espectrometria de massa e sequenciamento de exoma, a procura de alvos que sejam específicos para o tipo tumoral e não apresentam reatividade cruzada com antígenos próprios para que se evite uma reação de autoimunidade durante uma possível terapia antitumoral (GUBIN *et al.*, 2015; YARCHOAN *et al*., 2017).

Apesar da grande importância que se faz a busca por quais características definem um epítopo como um alvo imunogênico ou não responsivo para imunoterapia ainda continua sem explicação definitiva. Os estudos clássicos envolvendo epítopos se baseia em dados de sequência de aminoácidos, tais dados não levam em consideração fatores importantes para o desencadeamento de uma resposta imune, como a topologia e a distribuição eletrostática na molécula de MHC. Como alterações nas sequências de proteínas tumorais resultam em uma alteração estrutural que desloca a superfície eletrostática das moléculas de pMHC, a região crítica para o reconhecimento de TCR e o início de uma resposta imunogênica, reconhecer os padrões de distribuição eletrostática que conferem diferenças na imunogenicidade se torna uma peça fundamental para prospecção de novos alvos para imunoterapia, identificando quais alvos geram uma resposta imune satisfatória, auxiliando no desenvolvimento de vacinas e terapias envolvendo linfócitos T (RIGO *et al*., 2015; SINIGAGLIA *et al*., 2013).

Artigos previamente publicados já reportaram que padrões estruturais semelhantes estão relacionados a respostas similares no contexto de estudos envolvendo reatividade cruzada. Levando em consideração que foi evidenciado em trabalhos anteriores notaram que alterações

no potencial eletrostático na superfície do pMHC são impactantes para o reconhecimento do linfócito T tanto quanto, em menor magnitude, mudanças topológicas. Portanto, é factivel imaginar que comlexos com similaridades tanto eletrostáticas quanto topológicas podem ser reconhecidos pela mesma população específica de células T (ANTUNES *et al*., 2011; ANTUNES *et al*., 2017).

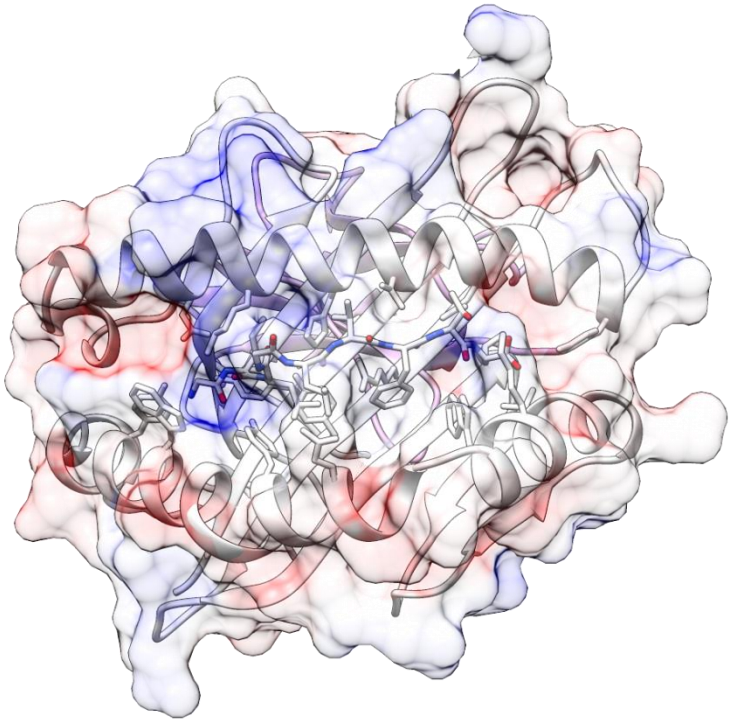

Figura 2. Uma representação esquemática de um complexo pMHC posicionado com a região analisada em evidência. Imagem gerada através do Software UCSF Chimera. Fonte: Elaborada pelo autor

1.5 JUSTIFICATIVA

Diversos trabalhos científicos tentaram compreender os fatores desencadeadores da resposta imune no contexto de câncer, entretanto, os reais fatores continuam sem uma explicação clara. Estudos sequências aparentam não dar uma resposta satisfatória. Isso se deve ao fato de que os reais elementos responsáveis pelo desencadeamento da resposta não estão restritos aos aminoácidos que compõem as sequências dos epítopos, mas sim, aos componentes moleculares decorrentes da combinação destes com os átomos da fenda do MHC. Sendo que essa interface de interação é que define se haverá ou não a formação da sinapse imunológica. A importância deste estudo se dá ao fato de que os fatores que definem se um peptídeo é imunogênico ou não responsivo continuam pouco elucidados. Estudos aprofundados sobre como a resposta imune é desencadeada se tornam crucial para o desenvolvimento de terapias celulares de precisão, que por sua vez, são uma das melhores apostas da medicina moderna para o tratamento de neoplasias com eficiência e baixa toxicidade quando comparada com a quimioterapia clássica.

1.6 OBJETIVOS

1.6.1 Objetivo geral

Identificar, através de análises de perfil eletrostático e técnicas de agrupamento hierárquico, padrões e/ou alterações de distribuição de carga em neoantígenos que estejam associados a um ganho de imunogenicidade.

1.6.2 Objetivos específicos

São objetivos específicos do trabalho:

a) Comparar as características moleculares envolvidas na apresentação e reconhecimento dos antígenos nos pares selvagens/mutados dos alvos, que pudessem explicar a variação na imunogenicidade;
b) Avaliar semelhanças em propriedades físico-químicas nos neoantígenos analisados com outros peptídeos virais previamente reportados como imunogênicos;
c) Incluir alvos previamente categorizados como não imunogênicos para avaliar se os padrões estruturais eram distintos do subconjunto imunogênico.

PLOS ONE

2 ARTIGO CIENTÍFICO

RESEARCH ARTICLE

Investigating the structural aspects that confer differential immunogenicity in tumoral T cell epitopes

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Abstract

The search for what characteristics define an epitope as either an immunogenic or a non-responsive target for immunotherapy has eluded researchers for years. Several studies demonstrate that certain positions in the peptide sequences, the MHC anchor residues, have a preferential composition of amino acids (allelic motifs), being those epitopes more likely to display a better immunogenic response. First of all, not all MHC ligands are immunogenic, considering that we have unnumbered self-epitopes being continuously presented in the cell surfaces. In this specific work, we tested an additional element, central in our hypothesis that alterations in tumor protein sequences result in a structural change that shifts the electrostatic surface of the pMHC molecules, pivotal for TCR recognition and the initiation of an immunogenic response. Then, previously neoepitope sequences presenting differential immune responses when compared with their wild-type counterpart were recovered. Despite the fact that the sequences were very similar, they triggered responses that were considerably different, and currently, there is no well-established explanation of why they conspicuously differ in immunogenic aspects to each other. pMHCs structures harboring the epitope sequences were modeled and then used to generate images of their electrostatic surfaces, looking for qualitative differences that can indicate the distinct responses. We noticed that no significant alteration occurred between immunogenic tumor peptides and their wild-type non-immunogenic counterparts when comparing their electrostatic surface. An additional comparison was made against structures of pMHCs containing immunogenic epitopes recovered from the Crosstope Database (www.crosstope.com). In this sense, it was also possible to verify if immunogenic tumor epitopes were similar to viral immunogenic ones. Surprisingly, both WT sequences and neoepitopes shared an electrostatic surface distribution with pathogen targets, which could be an indicative of their immunogenic predisposition. So we theorized that a “hidden element”, may be responsible for the immunogenicity shift in neoepitopes.

Introduction

1.2 The Immune system and the cytotoxic response

The Immune System identifies its targets through the recognition of molecular signatures presented by a specific pathogen [1]. The recognition process identifies molecular patterns that are present in pathogens/microbes but not found in host cells [2]. Innate immune recognition depends on receptors with broad specificity and can potentially bind to a large number of molecules that share a similar structure or have the same molecular pattern [3]. The adaptive immune response, similar to the innate system, works through mechanisms of recognition of self and non self; such identification by the adaptive system is based on the route of antigen presentation [4]. Our cells that are not infected or mutated have self-antigens that are different from those on the surface of bacteria or those found in virus-infected host cells, which normally, would classify as non-self [5].

Being part of the adaptive immune response, the T CD8 lymphocytes are the cells responsible for eliminating intracellular pathogens, such as viruses and abnormal cells, such as cancer cells. Through the interaction of the peptide-associated MHC molecule (pMHC) and the TCR, they are able to recognize viral particles presented by the MHC I surface molecule in the event of a viral infection and it is also capable of detecting mutated proteins generally exhibited in cancer The process responsible for the assembly of these complexes begin in the cytosol by the cellular machinery that transport proteins to the endoplasmic reticulum by the Transporter Associated with Antigen Processing (TAP), where it is associated with other proteins to form the Peptide Loading Complex. In this complex, assembly and loading of Type I MHCs occurs with the aid of the Tapasin proteins and culminates in the insertion of the peptide into the MHC cleft. From the docking event, the affinity between peptide and MHC dictates the completion of the process: if the affinity is low, the peptide will escape the cleft and the MHC will enter a "take-off" route; and if the affinity is high enough, the complex will emerge to the cell surface and will be presented [6,7].

Cancer: A brief review

One of the most prominent features in cancer is the high mutation rate that usually gives neoplastic cells the ability to adapt quickly to various conditions, such as hypoxia and lack of nutrients, it also confers the cell with tools necessary to maintain replication constantly until the cells acquire the means to spread to other regions of the body in a process known as metastasis [8,9]. The process to acquire new critical characteristics for the survival of cancer cells sometimes leads to the formation of mutant proteins called Neoantigens [10].

Neoantigens, or tumor-specific antigens (TSA), are proteins that are not expressed in normal cells and are the results of mutations in protein coding sequences. Neoantigen formation may occur during

neoplastic transformation itself or because of increased genetic instability at non-critical locations in the genome (in the form of transient mutations), which culminate in the formation of altered protein products. The development of therapies that could destroy tumors accurately without damaging healthy tissues, such as classical chemotherapy, has become one of the major challenges of oncology. Currently, immunotherapy has been a major therapy candidate to eliminating cancer cells with precision, avoiding damaging healthy cells. Although imperfect, immunotherapy is showing great promise; with its biggest challenge being the identification of the ideal targets for it. In this context, the advantage of the use of neoantigens as targets for immunotherapy is that the epitopes generated by the mutated proteins have not been subjected to thymic selection and central tolerance, so the organism must recognize these sequences as foreign and develop an immunogenic response against them [11,12,13].

1.4 Cancer and the Immune System: The rationale behind immunotherapy

In neoplastic development, when the immune system detects the anomaly and initiates a response, it usually divides into three stages: elimination, equilibrium, and escape. At first, the immune system is able to effectively eliminate aberrant cells and many aberrant growth starts usually end at this stage. If neoplastic cells escape this process, the equilibrium stage begins, which is characterized by the immune system keeping the neoplastic cells in a state of quiescence or functional dormancy. They remain in this state due to the constant immunological pressure and remain in this state for quite varied periods, and some evolve cellular variants capable of escaping the immune control, characterizing the escape phase. Through various mechanisms such as loss of MHC I molecules on the surface, increased signs of cell survival, development of an immunosuppressive tumor microenvironment and other mechanisms, tumor growth may escape the control of the immune system and begin to form a malignant tumor mass [14,15]. Assuming that the repertoire of tumor antigens is composed in part of mutated proteins of their own and are then recognized as immunogenic by the immune system, it was hypothesized that it would be possible to develop vaccines against neoantigens specifically expressed by a certain type of cancer [16, 17]. Nevertheless, for a tumor to effectively have a neoepitope repertoire that can be recognized by T cells, it is expected that the tumor possesses a sufficient mutation load on its genome. In another words, there is a correlation between the number of mutations on the genome and the formation of neoantigens [18].

In the field of innovative therapies for malignant tumors, the engineering of cells with chimeric receptors has been one of the most prominent techniques and with quite satisfactory results. Some of the most recent approaches includes the adoptive T cell immunotherapy, using tumor infiltrating lymphocytes (TILs), whose TCR is capable of recognizing tumor-associated antigens (TAA), and T lymphocytes with engineered receptors, also known as CAR-T cells. The Chimeric Antigen Receptor T-Cell (T-cell) cells have their original receptor replaced by a construct containing the variable chain region of an antibody fused to the TCR signaling chains, thus, these cells have the ability to recognize

multiple antigens in a non-restricted manner only by the peptide contained in the cleft of the MHC molecule, with the advantage of preserving all cytotoxic activity of a normal T lymphocyte. So far CARs have produced extraordinary results in several trials [19].

1.5 The structural aspect of neoantigens in the context of type I HLAs

Several studies have shown that proteins mutated in the context of cancer may exhibit sequences of epitopes capable of eliciting an immune response. Considering the efficiency of numerous clinical trials involving immunotherapies, the identification of new targets for T-cell precision therapies became pivotal for the development and effective application of such treatments in a wide scale. Locating the best targets through techniques such as mass spectrometry and exome sequencing, the demand of targets that are specific to the tumor type and do not cross-react with their own antigens so as to avoid an autoimmunity reaction during possible anti-tumor therapy [20,21].

Despite the great importance of the search for which characteristics define an epitope as an immunogenic or nonresponsive target for immunotherapy, it still remains without definitive explanation. Classical studies involving epitopes are based on amino acid sequence data, such data do not take into account factors important for triggering an immune response, such as structural elements of MHC and TCR molecules. For example, changes in tumor protein sequences can impact the electrostatic surface of pMHC molecules, the critical region for the recognition of TCR and triggering of lymphocytes [22,23]. So, the recognition of these patterns conferring differences in immunogenicity could represent a key element in the prospection of new targets for immunotherapy, identifying which targets generate a satisfactory immune response.

Taking into account what was evidenced in earlier works, that changes in the electrostatic potential on the surface of pMHC are relevant for T lymphocyte recognition as much as topological changes, albeit the latter to a lesser extent. Therefore, it is feasible to imagine that complexes with both electrostatic and topological similarities can be recognized by the same specific population of T cells [22, 23]. Considering the importance of a deeper understanding of what characteristics define an epitope as responsive and probably a good target for immunotherapy, we propose that the underlying cause of changes in immunogenicity between neoepitopes and their wild-type counterparts might rest in changes in the above presented molecular features.

Methods

Data set

The initial criteria utilized to prospect the targets to test our hypothesis were that all antigen selected must be presented in a context of HLA-A*02:01, have a length of nine amino acids, had its immunogenicity accessed through measurements of Interferon Gamma (IFNγ) or Chromium Release

assays and that it presented experimental data from both the mutated version of the peptide and its wild-type variant.

Molecular Modelling

Those antigens were then submitted to the Docktope tool [24], where the structure of the pMHC was generated and subsequently used to calculate the electrostatic data through Delphi [25] and GRASP 2.0 [26] to generate the image of the surface. This process was done for each and every individual model. Each pair of wild-type and Mutated images obtained were then compared qualitatively to determine if the change in amino acids caused by the mutation resulted in a significant change in the electrostatic surface. To rule out the possibility of the changes in immunogenicity be caused by shifts in the affinity between the peptide and the MHC molecule, we predicted the affinity of every target utilizing the NetMHC 4.0 software.

Comparing Tumor and Viral Epitopes

In a second moment, we analyzed two sets of electrostatic surface images, one containing the initial recovered dataset (mutated and immunogenic peptides) and the other was constituted of four tumor peptides that, although all harbored mutations on their sequences, did not trigger an effective immune response. We compared this dataset with the Crosstope [27] database to observe if the immunogenic neoepitopes shared electrostatic similarities with other viral epitopes previously reported as responsive.

After an initial visual inspection, we selected from two to three similar putative targets from the Crosstope database and compared them with our tumoral dataset in a hierarchical clustering to avoid any comparison bias. The input to feed the HCA was the numerical information from electrostatic potential distribution. The images of both the Crosstope complexes and the prospected targets were analyzed with the ImageJ software [28] by setting seven gates in regions close to the center of each image, which is the region where the epitope is presented. The information of positivity and negativity was recovered through readings of RGB (Red, Green, Blue), with a strong blue color representing a positive charged region while a strong red color represents a negative charged region. The data retrieved of each gate was the RGB values of mean, mode and standard deviation. After the selection process, the selected targets from Crosstope and the prospected targets from the literature were submitted to a hierarchical clustering with the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) parameters in the R Studio software [29]. This approach involves the evaluation of the distances measures through a matrix of dissimilarity based on correlation, and to compute covariances in the presence of missing values, it was used a pairwise complete observations and the results were reinforced in each branch though a bootstrap analysis replicated 10.000 times.

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Results and Discussion

Screening for the immunogenic neoantigens

To begin the testing, we searched in the literature for scientific articles that fulfilled the criteria previously established for our prospection. In a first moment, they were organized and had their affinities accessed to check if the changes in the immunogenicity were caused exclusively by the affinity's shift (Table 1).

Gene	Sequence (WT/MUT)	Affinity (nM) - WT	Affinity (nM) - MUT	Author
CLPP	ILDKVLVH(P/L)	6016.24	55.84	Corbière, 2011 [30]
ME1	FLDEFME(A/G)V	2.75	2.69	Karanikas, 2001 [31]
ERBB2	ALIHHNT(H/Y)L	79.25	17.86	Cohen, 2015 [32]
GANAB	ALYG(S/F)VPVL	15.28	8.25	Cohen, 2015 [32]
WDR46	FL(T/I)YLDVSV	6.45	4.02	Cohen, 2015 [32]
AHNAK	(S/F)MPDFDLHL	22.86	5.49	Cohen, 2015 [32]
NSDHL	ILTGLNYE(A/V)	41.68	7.45	Cohen, 2015 [32]
SRPX	T(P/L)WCSPIKV	12441.84	10.08	Cohen, 2015 [32]
HAUS3	ILNAMI(T/A)KI	53.01	48.08	Robbins, 2013 [33]
CSNK1A1	G(S/L)FGDIYLA	890.98	6.71	Robbins, 2013 [33]
*CRISPLD1**	CMQAN(P/S)HYA	383.72	326.53	Robbins, 2013 [33]
*WDR47**	MLFLRF(R/C)YI	84.75	22.62	Robbins, 2013 [33]
*CAMKK2**	RMLDKNPE(S/V)	1614.57	21.06	Robbins, 2013 [33]
*UNC13A**	SVVDVF(S/F)QL	679.22	65.56	Robbins, 2013 [33]

Table 1. A table containing all the prospected targets utilized in this analysis. The table holds the name of the gene which originated both sequences; its wild-type and its mutated nonamers sequences respectively; the affinities of the non-mutated and mutated versions of that gene, which were accessed through the NetMHC 4.0 software; and finally, the author of the article were these sequences were described and tested. All the sequences had a notable increase in its immunogenic capabilities after the mutation occurred, with the exception of the genes marked with an asterisk symbol.

After analyzing the affinities it was found that just 3 out of the 10 cases, that was reported a gain of immunogenicity, could have this change attributed to a shift in affinity (Fig. 1); while the other 7 cases, the wild-type sequences already were binders to HLA-A*02:01 allele (Fig. 2), so it is unlikely that this alteration was sufficient to be the main cause of the change in immunogenicity. We then progressed in our analysis to see if the mutations caused a significant shift in the electrostatic surface and to see if those said changes would cause enough variation to be a possible and visual explanation to this phenomenon of differential immunogenicity, especially in the cases not explained by binding affinity improvement. Notably, all the analyzed cases did not show any significant change in its electrostatic surface.

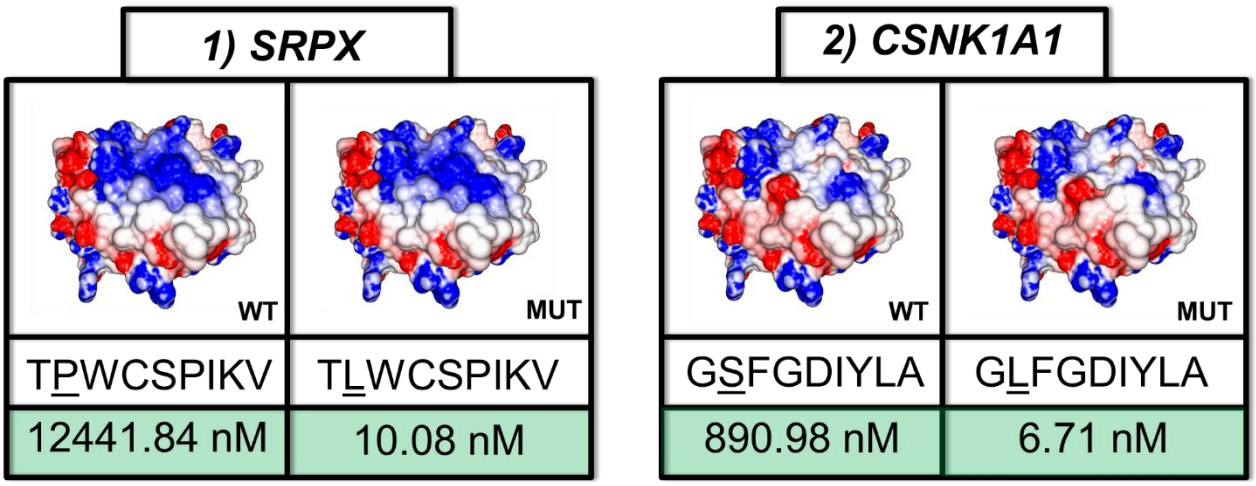

Fig 1. The electrostatic surface image of epitopes, both mutated and wild type. A figure showing a side-by-side view of the modeled complexes displaying their electrostatic surfaces, their sequences, both the wild-type and the mutated versions, with an underlining where the mutation occurred and their respective affinities in nM (nanoMolar) which was obtained through the software NetMHC 4.0. In the case of the gene *SRPX* and *CSNK1A1*, both presented nonamer sequences that, when mutated, had an exceptional gain in affinity, which could be a plausible explanation for the contrasting immunogenicity.

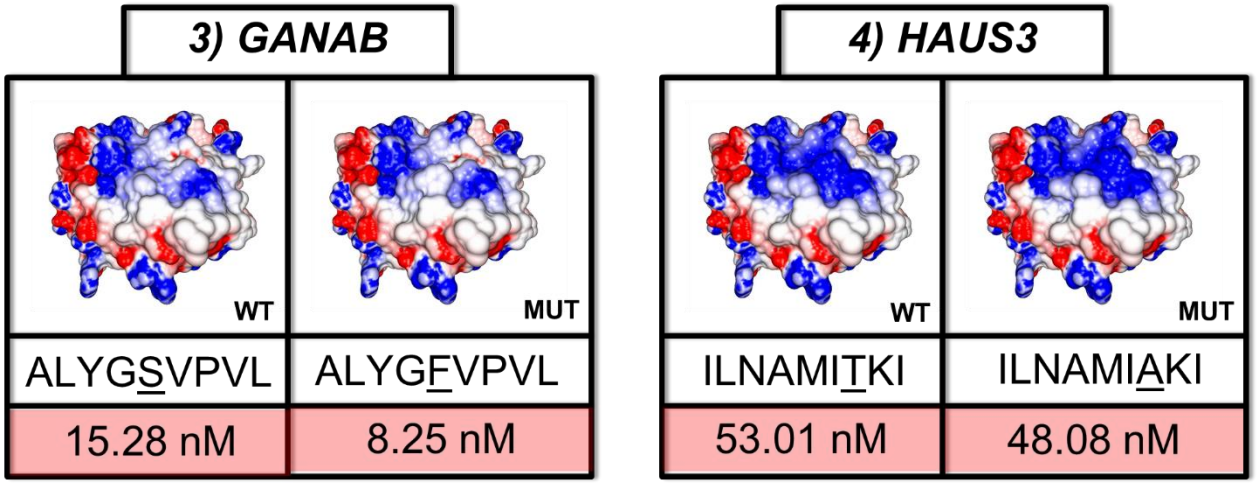

Fig 2. The electrostatic surface image of epitopes, both mutated and wild-type. A figure showing a side-by-side view of the modeled complexes displaying their electrostatic surfaces, their sequences, both the wild-type and the mutated versions, with an underlining where the mutation occurred and their respective affinities in nM (nanoMolar) which was obtained through the software NetMHC 4.0. In the case of the gene *GANAB* and *HAUS3*, both presented nonamer sequences that, when mutated, had no significant gain in affinity, leaving this initial analysis with no current explanation for the contrasting immunogenicity.

The analysis proceeded to verify if the prospected targets resembled previously known immunogenic targets from several viral epitopes to observe that, based on the cross-reactivity phenomenon, they harbor the immunogenic fingerprints that elicit an immune response [24, 34]. After verifying every epitope described in the Crosstope database for the HLA-A*02:01 allele we recovered many complexes presenting some level of structural identity with the cancer epitopes. For each tumor peptide we elected from two to three of Crosstope pMHCs complexes based in electrostatic similarities to perform a Hierarchichal Clustering Analysis. The results where then plotted in cladogram (Figure 3).

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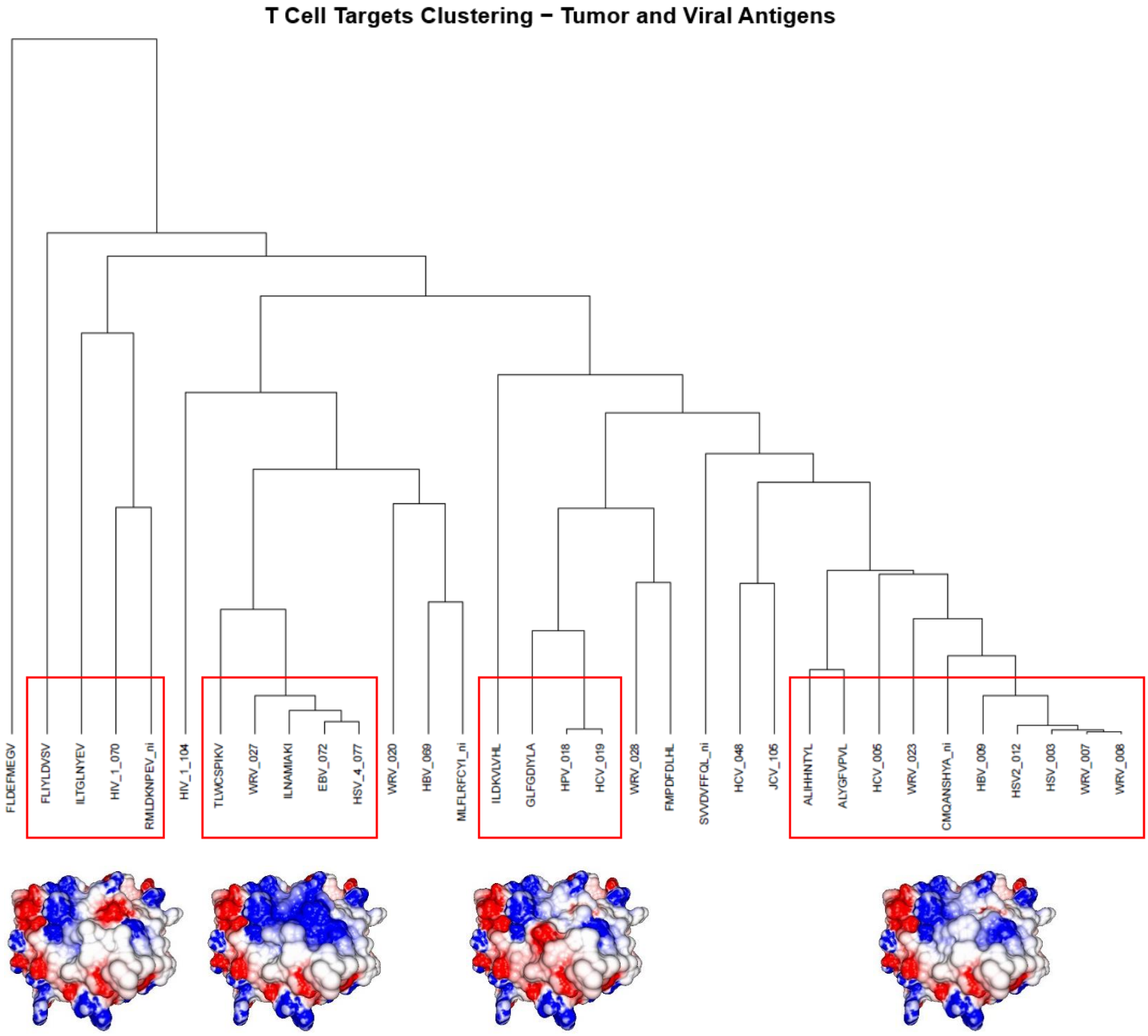

Fig 3. The HCA containing representatives of epitope clusters. A figure showing the results generated through the HCA. It contains all the prospected targets, both the ten highly immunogenic and the four low immunogenic targets, with the low immunogenic targets having their sequence followed by a "NI", which stands for "Non-Immunogenic. The cladogram also contains Crosstope structures, which can be identified as having an abbreviation of the virus those sequences derived, followed by the Crosstope code for each HLA-A*02:01 epitopes that clustered with the prospected targets. The viruses that grouped with the cancer targets are: Human Immunodeficiency Virus (HIV_1), Vaccinia Virus WR (WRV), Epstein-Barr Virus (EBV), Herpes Simplex Virus (HSV), Herpes Simplex Virus type 4 (HSV_4), Hepatitis B Virus (HBV), Human papillomavirus (HPV), Hepatitis C Virus (HCV), John Cunningham virus (JCV) and Herpes Simplex Virus type 2 (HSV2). The electrostatic surfaces below serve as a "representative" structure of each cluster segregated by the red boxes, having been separated by their difference in charge distribution around the surface.

In a first moment, it was noted that every individual structure inside each cluster possess subtle differences in the charge distribution, but still retained shared intracluster features, usually by having the same large concentrations of negative or positive charges on certain locations. Surprisingly, the three groups that comprised this analyses: The Crosstope, structures, the prospected immunogenic targets and the non-immunogenic targets were grouped evenly in the cluster. Considering this data, it is interesting to note that targets originated from cancer, mostly Melanoma, had similarities with viral epitopes and quite possibly, an immune response of the same magnitude – demonstrating a degenerate aspect of the immune system, as the cytotoxic response does not differentiate a virus infected cell from a tumor cell in the context of TCR recognition. In regards to the cases where epitopes with reported low release of Interferon Gama (therefore, considered non-immunogenic) that grouped with other known immunogenic targets, one of the most plausible explanation is that those epitopes are actually immunogenic, but the patient's lymphocytes did not carry a TCR capable of binding properly to the MHC carrying those neoepitopes, leading to a low immunogenic response. This hypothesis is supported by the fact that all non-immunogenic/low responsive derived from the same patient and were not similar to one another, so it is conceivable to speculate that the patient simply did not recognized those patterns, even though they just might be responsive. Also, the immunogenic epitopes ILNAMIAKI and GLFGDIYLA that originated from the same cell line did not group with the non-immunogenic targets, further corroborating to the idea that, during the selection stages in the thymus, a TCR capable of recognizing those surfaces was simply not conceived.

Conclusion

After analyzing the results, we could theorize that the fate of an immune response is not dictated simply by changes in affinity or shifts in the electrostatic surface. Taking into consideration that the studies with these epitopes involved T2 cells, it is unlikely that steps prior to the presentation of the pMHC are relevant to the outcome of the immune response. So we suggest that a "hidden element" is the one responsible for the difference in immunogenicity when neither the electrostatic surface nor the affinity can possibly explain.

Author Contributions

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3 CONCLUSÕES E PERSPECTIVAS

Depois de analisar os resultados, podemos teorizar que o destino de uma resposta imune não é ditado simplesmente por mudanças na afinidade ou mudanças na superfície eletrostática. Levando em consideração que os estudos com esses epitopos envolveram células T2, é improvável que etapas anteriores à apresentação do pMHC sejam relevantes para o resultado da resposta imune. Assim, sugerimos que um "elemento oculto" é o responsável pela diferença na imunogenicidade quando nem a superfície eletrostática nem a afinidade podem explicar.

Uma das possibilidades sobre o que pode estar provocando a diferença na resposta imune nestes alvos seria a estabilidade diferencial dos peptideos com a fenda do MHC. Assim, mesmo ambos possuindo uma afinidade suficientemente alta para que ocorra o evento de "encaixe", isso não garante que o peptideo eventualmente se dissocie da fenda, impedindo que a resposta imune ocorra normalmente. Levando isso em consideração, uma ferramenta capaz de acessar dados de estabilidade seria de grande relevância para este campo, pois ajudaria a determinar o quanto de fato a estabilidade impacta no desfecho de uma resposta imune.

Levando isso em conta, nossa perspectiva é poder ampliar o banco de dados utilizado neste estudo para que contenha mais estrturas conhecidamente imunogênicas, para que seja possivel extrair padrões relacionados com uma resposta eficiente e comparar com possíveis novos alvos quando analisando dados provenientes de tumores e/ou virus, e poder determinar qual população de células T seria a mais apropriada para uma terapia celular adotiva ou, em outra perspectiva, pensar no desenvolvimento de uma vacina carrgeando um antigeno que gere um epitopo que possua caracteristicas conhecidamente imunogênicas, garantindo uma proteção a longo prazo.

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA PLOS ONE

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Equations	We recommend using MathType for display and inline equations, as it will provide the most reliable outcome. If this is not possible, Equation Editor or

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Species names	Write in italics (e.g., *Homo sapiens*). Write out in full the genus and species, both in the title of the manuscript and at the first mention of an organism in a paper. After first mention, the first letter of the genus name followed by the full species name may be used (e.g., *H. sapiens*).
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Include a full title and a short title for the manuscript.

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Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

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- Summarize the most important results and their significance
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The introduction should:

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- Include a brief review of the key literature
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The Materials and Methods section should provide enough detail to allow suitably skilled investigators to fully replicate your study. Specific information and/or protocols for new methods should be included in detail. If materials, methods, and protocols are well established, authors may cite articles where those protocols are described in detail, but the submission should include sufficient information to be understood independent of these references.

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These sections may all be separate, or may be combined to create a mixed Results/Discussion section (commonly labeled "Results and Discussion") or a mixed Discussion/Conclusions section (commonly labeled "Discussion"). These sections may be further divided into subsections, each with a concise subheading, as appropriate. These sections have no word limit, but the language should be clear and concise.

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Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Source	Format
Published articles	Hou WR, Hou YL, Wu GF, Song Y, Su XL, Sun B, et al. cDNA, genomic

Source	Format
	sequence cloning and overexpression of ribosomal protein gene L9 (rpL9) of the giant panda (*Ailuropoda melanoleuca*). Genet Mol Res. 2011;10: 1576-1588. Devaraju P, Gulati R, Antony PT, Mithun CB, Negi VS. Susceptibility to SLE in South Indian Tamils may be influenced by genetic selection pressure on TLR2 and TLR9 genes. Mol Immunol. 2014 Nov 22. pii: S0161-5890(14)00313-7. doi: 10.1016/j.molimm.2014.11.005. *Note: A DOI number for the full-text article is acceptable as an alternative to or in addition to traditional volume and page numbers. When providing a DOI, adhere to the format in the example above with both the label and full DOI included at the end of the reference (doi: 10.1016/j.molimm.2014.11.005). Do not provide a shortened DOI or the URL.*
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Books	Bates B. Bargaining for life: A social history of tuberculosis. 1st ed. Philadelphia: University of Pennsylvania Press; 1992.
Book chapters	Hansen B. New York City epidemics and history for the public. In: Harden VA, Risse GB, editors. AIDS and the historian. Bethesda: National Institutes of Health; 1991. pp. 21-28.
Deposited articles (preprints, e-prints, or arXiv)	Krick T, Shub DA, Verstraete N, Ferreiro DU, Alonso LG, Shub M, et al. Amino acid metabolism conflicts with protein diversity; 1991. Preprint. Available from: arXiv:1403.3301v1. Cited 17 March 2014.
Published media (print or online newspapers and magazine articles)	Fountain H. For Already Vulnerable Penguins, Study Finds Climate Change Is Another Danger. The New York Times. 29 Jan 2014. Available from: http://www.nytimes.com/2014/01/30/science/earth/climate-change-taking-toll-on-penguins-study-finds.html Cited 17 March 2014.
New media (blogs, web sites, or other written works)	Allen L. Announcing PLOS Blogs. 2010 Sep 1 [cited 17 March 2014]. In: PLOS Blogs [Internet]. San Francisco: PLOS 2006 - . [about 2 screens]. Available from: http://blogs.plos.org/plos/2010/09/announcing-plos-blogs/.
Masters' theses or doctoral dissertations	Wells A. Exploring the development of the independent, electronic, scholarly journal. M.Sc. Thesis, The University of Sheffield. 1999. Available from: http://cumincad.scix.net/cgi-bin/works/Show?2e09
Databases and	Roberts SB. QPX Genome Browser Feature Tracks; 2013 [cited 2013 Oct 5].

Source	Format
repositories (Figshare, arXiv)	Database: figshare [Internet]. Available from: http://figshare.com/articles/QPX_Genome_Browser_Feature_Tracks/701214
Multimedia (videos, movies, or TV shows)	Hitchcock A, producer and director. Rear Window [Film]; 1954. Los Angeles: MGM.

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