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Self-mediated positive selection of T cells sets an obstacle to the recognition of nonself

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Publications not directly related to the thesis

II. Máté Manczinger, **Balázs Koncz**, Gergő Mihály Balogh, Benjamin Tamás Papp, Leó Asztalos, Lajos Kemény, Balázs Papp & Csaba Pál. Negative trade-off between neoantigen repertoire breadth and the specificity of HLA-I molecules shapes antitumor immunity. *Nature Cancer* (2021). DOI: 10.1038/s43018-021-00226-4.

III. Párniczky A, Lantos T, Tóth EM, Szakács Z, Gódi S, Hágendorn R, Illés D, **Koncz B**, Márta K, Mikó A, Mosztbacher D, Németh BC, Pécsi D, Szabó A, Szücs Á, Varjú P, Szentesi A, Darvasi E, Erőss B, Izbéki F, Gajdán L, Halász A, Vincze Á, Szabó I, Pár G, Bajor J, Sarlós P, Czimmer J, Hamvas J, Takács T, Szepes Z, Czakó L, Varga M, Novák J, Bod B, Szepes A, Sümegi J, Papp M, Góg C, Török I, Huang W, Xia Q, Xue P, Li W, Chen W, Shirinskaya NV, Poluektov VL, Shirinskaya AV, Hegyi PJ, Bátovský M, Rodriguez-Oballe JA, Salas IM, Lopez-Diaz J, Dominguez-Munoz JE, Molero X, Pando E, Ruiz-Rebollo ML, Burgueño-Gómez B, Chang YT, Chang MC, Sud A, Moore D, Sutton R, Gougol A, Papachristou GI, Susak YM, Tiuliukin IO, Gomes AP, Oliveira MJ, Aparício DJ, Tantau M, Kurti F, Kovacheva-Slavova M, Stecher SS, Mayerle J, Poropat G, Das K, Marino MV, Capurso G, Małecka-Panas E, Zatorski H, Gasiorowska A, Fabisiak N, Ceranowicz P, Kuśnierz-Cabala B, Carvalho JR, Fernandes SR, Chang JH, Choi EK, Han J, Bertilsson S, Jumaa H, Sandblom G, Kacar S, Baltatzis M, Varabei AV, Yeshy V, Chooklin S, Kozachenko A, Veligotsky N, Hegyi P; Hungarian Pancreatic Study Group. Antibiotic therapy in acute pancreatitis: From global overuse to evidence based recommendations. *Pancreatology* (2019). DOI: 10.1016/j.pan.2019.04.003. IF: 3.996, SCImago Journal Rank: Q1 / D3.

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INTRODUCTION

Basis of adaptive immune recognition

In the second half of the 20th century, the self-nonself theory was the general view in immunology, which suggests that the immune system's primary goal is to discriminate between self and nonself. Therefore, an immune response is triggered against foreign entities, but not against the organism's own materials. In the 1990s, *Matzinger* outlined the danger theory, which suggests that the immune system has a principal role in the danger detection and protection against harmful agents. It claims that self-elements can trigger an immune response if they are dangerous (e.g., cellular stress) and nonself constituents can be tolerated if they are not dangerous (e.g., commensal bacteria). In sum, if the immune system could effectively recognize cells that contain mutated proteins or intracellular pathogens, then there is a higher chance for it to destroy them.

The immune system is typically divided into two subsystems - innate and adaptive – which operate in various but coordinated ways. Innate immunity is ready for action from the very beginning of an infection. However innate immunity alone is unable to defeat most infections. Therefore, the adaptive immune system joins the battle and responds to the intruder in a very specific and more effective way. Antigens play a key role in the adaptive immune response. These molecules are originated from pathogens or are produced by human cells and can trigger an immune response. The adaptive immune response is specific for antigens and provides long-lasting immunity against pathogens.

T cell activation is dependent on the formation of the immunological synapse. The latter structure consists of the peptide-human leukocyte antigen (pHLA) complex, T cell receptor (TCR), adhesion molecules, and checkpoint receptors. HLA molecules have a major role in peptide presentation during the adaptive immune response. HLA genes are extremely polymorphic: more than 20,000 HLA alleles are registered currently. Normal and mutated self-peptides and the ones that are originated from intracellular pathogens are presented by HLA-I molecules which are expressed in almost all cells. At the same time peptides of extracellular proteins and extracellular pathogens appear on the cell surface bound to HLA-II molecules which are mainly found in B cells, myeloid dendritic cells and monocytes.

In my thesis, I am going to focus on HLA-I-presented peptides therefore I explain the intrinsic antigen presentation pathway in detail. Self or pathogen-associated proteins are cleaved to 8-11 (most often 9) amino acids long fragments by proteasomes and aminopeptidases in the cytosol.

HLA-I molecules bind peptides within the peptide loading complex. The resulting pHLA-I complex is transported to the cell surface, where it is anchored to the cell membrane. HLA-I presentation of peptides is highly dependent on the expression of the encoding gene.

Adaptive immune recognition is dependent on the presence of antigen-specific T cells in the T cell repertoire. If antigen-specific cytotoxic T cells (CTL) can be found in the repertoire, the immunological synapse can be formed. In a healthy state, the immune response is dependent on the peptides presented on the cell surface: tolerance is developed to cells presenting only self-peptides, and the immune system eliminates cells presenting foreign or dangerous peptides (infected cells, tumor cells). If there are no specific T cells in the T cell repertoire, the antigen-specific immune response is lacking.

T cell receptors recognize T cell exposed motifs of peptide sequences

In the peptide-HLA-TCR complex, some amino acids of the peptide have a critical role in HLA-binding (anchor residues) and are partially hidden from the TCR, while others are mainly responsible for TCR binding. A computational study described that in a nine amino acids long (9-mer) peptide positions 4-8 have a significantly higher number of interactions with TCRs than positions 1-3 and 9. Consequently in my analyses, I will focus on the *T cell exposed motif* (TCEM) which can be found between positions 4 to 8 of 9-mers as defined by *Bremel* and *Homan*.

The development of the T cell repertoire

The T cell repertoire is formed during fetal development, and it is already established at birth. Positive and negative selection processes are key to establish a functionally competent and self-tolerant T cell repertoire. Although TCRs are generated by a quasi-random process of somatic recombination, the mature T cell repertoire – the subset of all possible TCRs – is far from random, whereas the aim is to develop a T cell population that is effective in fighting pathogens. Selection processes are dependent on the presented self-peptides by thymic antigen-presenting cells.

T cell precursors (thymocytes) evolve to double-positive T cells expressing CD4 and CD8 coreceptors as a result of certain intracellular stimuli. During positive selection, these cells become CTLs if they recognize self-pHLA-I complexes on cortical thymic epithelial cells (cTEC). This encounter represents an essential signal for thymocyte survival and differentiation. T cells incapable of binding pHLA-I complexes *die by neglect*. It is important to emphasize that the TCR ligand pool is composed of self-pHLAs. cTECs exclusively express

a particular proteinase complex, called thymoproteasome, which produces unique peptide motifs for the positive selection of CTLs. Peptides generated by thymoproteasomes exhibit low affinity to TCRs and induce large fractions of CD8+ cells. A recent study published the amino acid preferences of the thymoproteasome around the cleavage site. Experiments confirmed that thymoproteasomes are essential for the positive selection of an adequate number of CTLs.

Positively selected T cells migrate into the medulla. During negative selection T cells expressing TCRs that bind self-pHLA ligands with high affinity die by apoptosis.

Although negative selection is highly efficient, the control of self-reactivity is not yet perfect. The essence of the peripheral tolerance processes is to provide additional control over self-reactivity: self-reactive T cells become functionally unresponsive (anergic) or are deleted after binding self-antigens outside of the thymus.

Immunogenicity of nonself peptides and cross-reactivity of T cells

How can T cells differentiate between self and nonself peptides, dangerous and harmless signs? T-cell responses to a given peptide are influenced by several factors. One of the most important factors is the similarity to self-antigens or commensal antigens. Numerous studies showed that peptides similar to self-antigens have lower immunogenicity, presumably as a result of the negative selection of T cells. TCRs are typically able to bind not just one particular peptide but a set of peptides (cross-reactivity of TCRs), which consists of closely related sequences. As T cells are cross-reactive, a significant fraction of nonself peptides is indistinguishable from presented self-peptides and T cells are missing from the repertoire or they are tolerant to these peptides. Note, the size of the peptide set (polispecificity) varies between TCRs.

At the same time, it is widely accepted that nonself peptides highly dissimilar to human proteins are more immunogenic.

AIMS

It has been suggested that as a result of T cell positive selection, both the CD4+ and CD8+ T cell repertoires are skewed to greater self-reactivity, and T cells that bind self-peptides stronger also bind the foreign agonist peptides more effectively. In other words, self-peptides mediating positive selection can be considered as a 'test-set' selecting T cells that recognize foreign peptides with higher effectivity. But is there any negative consequence of this mechanism?

Our hypothesis suggests a fundamental side-effect of T cell positive selection on the recognition of nonself peptides.

> As sequences of self-proteins mediate positive selection, a large fraction of nonself peptides is not recognized by the immune system even if T cells are cross-reactive.

As T cell positive selection is mediated by TCEMs of self-peptides, the hypothesis predicts that it is less likely to detect specific T cells in the repertoire for TCEMs that are 1) extremely rare or missing from human proteins, 2) not expressed in cTECs, or 3) not presented on the surface of cTECs.

Additionally, the hypothesis raises several questions. Is it possible that a peptide is overly different from self-proteins and consequently it is not recognized by T cells? Could T cell cross-reactivity compensate for this side-effect of T cell positive selection? Does this phenomenon have any effect on the susceptibility to infections? In my thesis, I aim to confirm the predictions of the hypothesis and answer the questions that arose.

METHODS

In vitro datasets

Our hypothesis was tested on two independent in vitro datasets. HLA-I associated peptides were collected from the Immune Epitope Database (IEDB). The final datasets were compiled using strict selection criteria. HLA binding is the prerequisite of T cell activation. Therefore, the HLA binding of peptides was confirmed by two alternative approaches:

(1) The binding between peptides and alleles was determined using the state-of-the-art bioinformatics tool, *NetMHCpan*. The general guidelines were used to select bound peptides (**dataset 1**).

(2) To avoid the bias associated with the computational prediction of HLA binding, HLA binding assays that were also collected in IEDB were matched with allele-peptide pairs of activation assays (**dataset 2**).

Previous works have suggested that the overrepresentation of highly similar sequences due to collection bias in the IEDB could influence the analysis results. Consequently, in the case of dataset 2, a highly diverse peptide set was created using a previously established iterative method, which excluded similar peptide sequences from peptides.

Peptides were classified into two groups: in both datasets, allele-peptide pairs with solely negative T cell assays were defined as **nonimmunogenic** and the ones with more positive than negative T cell assays as **immunogenic**. After filtering, the number of peptides in datasets 1 and 2 were 3,380 and 635, respectively.

TCEM frequency, TCEM expression and thymoproteasomal cleavage score

For each peptide, TCEM was defined as the amino acid sequence from positions 4 through 8 for 9-mers and the amino acid sequence between positions 5 and 9 for 10-mers. **TCEM frequency** in the human proteome was determined for each five amino acid-long sequences (pentamer) as the number of occurrences in the human reference proteome.

A recently published study reported the gene expression of human thymic cortical epithelial cells from infants. The median RPKM value in cTEC samples was determined for each gene. To assign an expression value to a TCEM, the proteins containing a given TCEM were collected. The median expression of genes encoding these proteins was calculated to approximate the chance for a given TCEM being expressed in cTECs (**TCEM expression**). TCEMs encoded by housekeeping genes were determined using data from a recent study.

A previous study reported the amino acid prevalence around the cleavage site of the thymo- and immunoproteasome. We developed a score, which estimated the probability of cleavage between two amino acids at a given amino acid environment. We approximated the probability of peptide formation upon proteasomal cleavage by implementing cleavage scores. Then for each pentamer, we calculated the median of values of all 9-mers that contain the given pentamer in the TCEM region yielding the **thymoproteasomal cleavage score**. We calculated the immunoproteasomal cleavage score using the same approach. This score served as a control in the analysis.

Sequence similarity score

Sequence similarity score was calculated using an established method. The similarity between a given peptide and the most similar sequence in the human reference proteome was estimated using the BLOSUM62 substitution matrix. First, the most similar peptides in the human proteome for a given peptide were found with *BLAST* software, and then the similarity score was calculated between each pair using an established formula.

SARS-CoV-2 specific T cells in the repertoire

Multiplex Identification of T-cell Receptor Antigen Specificity (MIRA) data on 27 healthy individuals were acquired from the website of Adaptive Biotechnologies. HLA-I allele-peptide pairs were determined, for which carrying of a given allele could potentially be associated with the prevalence of specific naive CD8+ T cells in the repertoire. For each patient, the expected peptides with specific T cells in the repertoire were determined.

The level of T cell cross-reactivity

Data on the binding strength of T cells were reported in two studies. Each study examined the shift in peptide-binding by TCRs when sequentially changing amino acids at each peptide position. The analysis was narrowed down to the TCEM sequence, and the BLOSUM62 similarity was determined between the TCEM of the original and the modified peptides. ROC curves and ROC AUC values were determined. In the case of the NY-ESO-1 epitope and TCR C^{259}, the level of TCR binding strength was determined, under which T cell activation is negligible. To identify this value, T cell activation data of the sequentially modified NY-ESO-1 epitopes (reported in the same study) were used. Lower than 10% of original TCR binding strength was selected as insufficient binding.

Determining TCEMs in intracellular pathogens and analyzing HLA association data

The reference proteomes of 50 well-known intracellular pathogens were downloaded from the UniProt database. First, the TCEMs of each 9-mer in the proteome of each pathogen and their prevalence in the human proteome, expression in cTECs, and the probability of proteasomal cleavage were determined as previously described. np-TCEMs were defined as the ones found less than 4 times in the human proteome or have low expression in cTECs or low probability of thymoproteasomal cleavage. Then, the binding of each 9-mer to common HLA alleles was predicted with *NetMHCpan*. For each allele-species pair, the fraction of np-TCEMs in bound peptides was calculated.

To identify HLA allele associations with infectious diseases, a literature mining was carried out. We focused on meta-analyses to collect highly reliable HLA associations. HLA association meta-analysis studies were found for hepatitis B, hepatitis C, dengue virus, and human papillomavirus. As the results of all studies were published for allele groups or serotypes and not individual alleles, the fraction of np-TCEMs in presented peptides was calculated as follows. For serotypes, the mean values of alleles belonging to the given serotype were calculated. In the case of allele groups (i.e., associations published for two digits resolution), the mean values of alleles were calculated, which are marked as common in the Common and Well-Documented Alleles Catalog.

RESULTS

Analysing the effect of T cell positive selection on peptide immunogenicity

The three predictions of the hypothesis were examined in parallel on two nonoverlapping in vitro T cell activation datasets. Dataset 1 contained a high number of peptides, while dataset 2 ensured that the findings are not confounded by computational prediction or the presence of similar sequences.

Our hypothesis predicted the following: if a TCEM is very rare in the human proteome, specific T cells will unlikely survive the positive selection around cTECs, and they will be potentially missing from the T cell repertoire. Consequently, motifs very rarely or not found in the human proteome are less likely to be immunogenic. Indeed, the TCEM frequency of immunogenic and nonimmunogenic peptides was significantly different in both datasets: immunogenic peptides contained more frequent TCEMs. The result suggests that an appropriate occurrence of TCEMs in human proteins is needed for immunogenicity.

Our hypothesis also predicted that TCEMs encoded by genes having low or undetectable expression in cTECs cannot mediate the positive selection of specific T cells. At the same time, the immune response is not expected to TCEMs that are encoded by abundantly expressed housekeeping genes, because the response to these TCEMs may be blocked by central or peripheral immune tolerance mechanisms. In line with our expectation, TCEMs having either low or high expression in cTECs were similarly less likely to activate T cells than the ones in the medium expression group.

In cTECs, a specific proteasome called the thymoproteasome generates most peptides from intracellular proteins for T cell positive selection. If a peptide is generated with a low probability after thymoproteasomal cleavage, it has a little chance to be presented on the cell surface in complex with HLA molecules even if it has a high expression in the cell. Our results show that TCEMs of immunogenic peptides were more likely to be generated by thymoproteasomal cleavage than nonimmunogenic ones, while immunoproteasomal cleavage did not affect immunogenicity.

These effects on immunogenicity held in multivariate logistic regression models indicating that they are not confounded by and independent of each other. Additionally, the effect of these attributes was additive: rare TCEMs having low expression in cTECs and low thymoproteasomal cleavage score were less likely to be immunogenic than TCEMs having only one or two of these attributes.

The frequency, expression, and presentation of TCEMs determine the prevalence of specific naïve CD8+ T cells in the repertoire

To confirm the previous findings, the predictions of the primary hypothesis were directly demonstrated on T cell repertoires of healthy individuals. Based on the hypothesis, it is less likely to detect a given naïve T cell in the repertoire that is specific for infrequent TCEMs in human proteins, for TCEMs not expressed in cTECs, or for TCEMs not presented on the surface of cTECs. Recently published data were utilized to demonstrate the absence of such T cells in the repertoire of healthy individuals. Specific naive CD8+ T cells were less likely to be present for rare than nonrare TCEMs in the repertoire of healthy individuals. Similarly, it was less likely to observe specific T cells for TCEMs having either negligible or overly high expression in cTECs. Moreover, TCEMs with low thymoproteasomal cleavage scores were less likely to be associated with the presence of specific T cells in the repertoire, while the immunoproteasomal cleavage score did not show this relationship. In sum, these findings on T cell repertoire data confirmed the ones on in vitro T cell activation data.

Decreased immunogenicity of overly dissimilar peptides to human proteins

The leading hypothesis predicted a rather provocative relationship: in contrast with expectation, overly dissimilar peptides are not recognized by the immune system, because self-peptides mediate the positive selection of specific T cells. Put differently, it is less likely to find TCEMs of highly dissimilar peptides in the human proteome and, thus, specific positively selected T cells are potentially absent from the repertoire. As expected, peptides with exceptionally rare TCEMs in the human proteome had lower similarity than other peptides. Accordingly, overly dissimilar peptides of datasets 1 and 2 were less likely to be immunogenic just like highly similar ones. To corroborate these results, the self-similarity of SARS-CoV-2 peptides was analyzed. Reassuringly, naive CD8+ T cells specific for highly dissimilar peptides were found in the repertoire of fewer individuals.

Cross-reactivity is not able to compensate for the side-effect of self-mediated positive selection of T cells

Our results suggest that the mechanism of positive selection results in a defective T cell repertoire. We found that T cells in the repertoire are unlikely to recognize TCEMs, whose recognition is negatively affected by self-mediated positive selection (i.e. TCEMs found less than 4 times in the human proteome, have low expression in cTECs and low thymoproteasomal cleavage score), so the cross-reactivity of TCRs is not able to compensate for these defects.

Positive selection of T cells and susceptibility to infections

The adaptive recognition of pathogen-associated peptide sequences is essential for the initiation of an effective immune response. Presented results suggest that many such sequences are potentially nonimmunogenic because specific T cells are not observed in the CD8+ T cell repertoire. In the proteome of 50 familiar intracellular pathogens, the prevalence of TCEMs was determined, that are either rare or not found in human proteins and/or not or lowly expressed in cTECs and/or unlikely to be presented after thymoproteasomal cleavage (called np-TCEMs). The frequency of these np-TCEMs ranged from 58% to 71% in different species.

This high fraction of np-TCEMs could hinder immune recognition, especially when only a few peptides of the pathogen are presented because either the proteome of the pathogen is small and/or the HLA allele has a narrow binding repertoire. To this end, the binding of all 9-mer peptides found in the proteome of pathogens was predicted to the most common HLA-I alleles. For each allele-species pair, the fraction of np-TCEMs was calculated in the presented peptides and as expected, the fraction of presented peptides with np-TCEMs was extremely variable between HLA alleles when the pathogens had small proteomes.

We expected an HLA-dependent effect of np-TCEMs on disease risk. To this end, HLA association meta-analysis data were collected. Allele groups with positive or negative associations were selected and the fraction of np-TCEMs presented by alleles in each group from all peptides of the causative pathogens were calculated. Allele groups associated with infections or treatment failure dominantly presented np-TCEMs in contrast with protective allele groups.

SUMMARY

The adaptive immune recognition is mediated by the binding of peptide-HLA complexes by T cells. Positive selection of T cells in the thymus is a fundamental step in the generation of a responding T cell repertoire: only those T cells survive that recognize human peptides presented on the surface of cortical thymic epithelial cells.

Three lines of evidence were reported suggesting that the self-mediated positive selection of T cells results in a defective T cell repertoire with implications on the recognition of nonself peptides. First, TCEMs that are very rare or not found in human proteins are less likely to be immunogenic. Second, the scarce expression of TCEMs in cTECs is also associated with lower immunogenicity. Third, TCEMs that are improbably generated by the cTEC-specific thymoproteasome are less likely to be immunogenic. Peptides carrying such motifs were especially dissimilar to human proteins. Importantly, we present our main findings on two independent T cell activation datasets and directly demonstrate the absence of naïve T cells in the repertoire of healthy individuals.

We also show that T cell cross-reactivity is unable to compensate for the absence of positively selected T cells.

Additionally, our results suggest that the proposed side-effect of T cell positive selection influences the adaptive immune recognition of intracellular pathogens and the risk for different infectious diseases.

In sum, our results suggest a side-effect of T cell positive selection, which could explain the non-responsiveness to many nonself peptides and could improve the understanding of adaptive immune recognition.

We conclude that while a given level of peptide dissimilarity to human proteins is essential for self-nonself discrimination, overly dissimilar peptides are less likely to be recognized by the immune system because specific T cells are not present in the repertoire.

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