

Identification of T-cell epitopes from benzylpenicillin conjugated to human serum albumin and implication in penicillin allergy

Short title: T-cell epitopes in penicillin allergy

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Abstract

Background: There is *in vitro* evidence that T-cells from allergic patients react to benzylpenicillin-human serum albumin (BP-HSA) bioconjugates. Our group has recently shown the existence of naïve CD4⁺ T lymphocytes recognizing BP-HSA in healthy donors. However, BP-haptenated peptides from HSA participating in the immunization of allergic patients have never been identified. The purpose of the present study is to identify immunodominant BP-haptenated peptides from HSA involved in immunization of patients to BP and to refine the frequency calculation of naïve CD4⁺ T-cells recognizing BP.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/all.13418

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Methods: Co-cultures were established with CD4⁺ T-cell from non-allergic donors and mature autologous dendritic cells (DCs) loaded with BP-HSA or BP-haptenated peptides from HSA. The CD4⁺ T-cell response specific for BP-HSA or for individual BP-haptenated peptides was measured using an interferon- γ (IFN- γ) ELISpot assay. Frequency of BP-specific CD4⁺ T-cell was then calculated using the Poisson distribution. BP-HSA and BP-haptenated peptides recognition by allergic patients was evaluated on peripheral blood mononuclear cells (PBMCs) using a lymphocyte transformation test (LTT).

Results: Results showed that BP-HSA and BP-haptenated peptides were recognized by naïve T-cells from 15/16 and 13/14 tested healthy donors respectively. Most donors responded to 3 peptides with BP covalently bound on lysines 159, 212 and 525. Two of these benzylpenicilloylated peptides (lysines 159 and 525) were also found to induce PBMCs proliferation in patients with allergic reaction to penicillins.

Conclusion: This study identifies and characterizes for the first time the BP-haptenated peptides from HSA involved in the immunization of patients to penicillins.

Keywords:

Allergy; drug allergy; hapten; penicillin; T-cell

Abbreviations:

AX, amoxicillin; BP, benzylpenicillin; CLV, clavulanic acid; DCs, dendritic cells; F, female; HLA, human leukocyte antigen; HSA, human serum albumin; IFN- γ : interferon- γ ; KLH, Keyhole limpet hemocyanin; LTT, lymphocyte transformation test; M, male; MDM, minor determinant mixture; MHC, Major histocompatibility complex; MS, mass spectrometry; PBMCs, Peripheral blood mononuclear cell; PHA, phytohemagglutinin; PPL, benzylpenicilloyl-polylysine (major determinant); SK, streptokinase; TCR, T-cell receptor; yo, years old.

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Introduction

Penicillins are considered the drugs most frequently eliciting allergic reactions^{1,2}. Patients commonly complain of various and heterogeneous symptoms ranging from immediate skin rash, respiratory symptoms and anaphylaxis, to more delayed reactions such as bullous skin eruptions³. The mechanisms of immediate reactions to penicillins are mediated by specific IgE antibodies and penicillin-specific CD4⁺ T-cells³.

Spontaneous protein modification by the reactive form of the drug is an important process in adverse drug reactions⁴. In this process, drugs or haptens bind covalently to protein to form a complex of a sufficient size to trigger an immune response (haptenation)⁵⁻⁷. This structure can later be processed by antigen presenting cells⁴, such as dendritic cells (DCs), and the resulting haptenated peptides exposed through major histocompatibility complex (MHC) class I or class II-dependent pathways are presented to T-cell receptors (TCRs). The specific amino acid sequence of peptides determines MHC allele specificity and the nature of the response^{8,9}. Alternative mechanisms imply the covalent or non-covalent binding (P-i concept) of the drug to the peptides already exposed on the MHC^{10,11}. From a chemical point of view, the reactivity of penicillins depends on the β -lactam ring electrophilic character which suffers a nucleophilic attack by the amino groups of the lateral chains of lysine residues, resulting in the opening of the β -lactam structure¹². Early works on the detection and identification of β -lactam-protein adducts allowed the identification of several proteins targeted by penicillins including human serum albumin (HSA), transferrin and immunoglobulins^{13,1}. Penicillin binding sites on HSA have been found both *in vivo* using sera obtained from patients and *in vitro* by synthesizing bioconjugates all of which mapped at lysine residues^{1,14-16}. We have recently used a combination of immunological and high-resolution MS (Mass Spectrometry) approaches to identify Benzylpenicillin (BP) binding sites on HSA¹⁷.

There is *in vitro* evidence that T-cells from allergic patients react with BP-HSA bioconjugates¹². Our group has recently shown the existence of naïve CD4⁺ T-cells specific for BP-HSA in healthy donors¹⁸. Moreover, activation of T-cell clones from allergic patients may occur selectively in response to free drug or to drug conjugates^{1,19}. This latter observation raises the hypothesis that not only the drug, but also parts of the haptenated protein or peptides coupled to the drug may constitute the important structural determinants for antigen recognition by the TCR.

In this context, we were interested in defining candidate haptenated peptides from circulating proteins that are able to immunize patients to BP leading to hypersensitivity reactions. HSA is a good candidate for BP haptenization since BP-HSA conjugates have been identified in allergic patients. We selected candidate peptides using an *in silico* approach addressing peptide binding to HLA molecules. BP-haptenated peptides were synthesized using an original BP-lysine monomer¹⁷. With the selected haptenated peptides, we were able to define BP-haptenated peptides recognized by naïve T-cells. BP-HSA and BP-haptenated peptides were then tested in penicillin-allergic patients showing significant specific lymphocyte proliferation to BP-HSA and to the selected BP-haptenated peptides previously found positive with naïve T-cells.

Methods

Synthesis of benzylpenicillin-human serum albumin

BP-HSA was produced as described previously^{18,19}. The presence of BP on HSA was confirmed using Western-blots and mass spectrometry MALDI-TOF analysis and is published in a companion work¹⁷. BP binding sites on HSA were identified through tryptic digestion of BP-HSA bioconjugates followed by MALDI-MS and nano-LC-MS/MS analysis.

Benzylpenicillin-haptenated peptides

Twelve 15 mer long BP-haptenated peptides identified as potential T-cell epitopes were selected using the MHC II binding prediction tool available at the IEDB (www.iedb.org)^{17,20,21}. A percentile rank was generated allowing a uniform comparison of peptide's binding affinity to HLA alleles across different predictors. A peptide was considered a good binder for a specific allele if its IEDB predicted consensus percentile rank was ≤ 20 ²². BP-haptenated peptides were synthesised using a BP-lysine monomer¹⁷ and standard Fmoc chemistry. They were purified by RP-HPLC to approximately 90 % purity as determined by analytical HPLC. Non-haptenated peptides were also synthesized as negative controls. Sequences, annotations and lysine positions of BP-haptenated peptides are shown in supplementary data S1.

Generation of benzylpenicillin-human serum albumin and benzylpenicillin haptenated peptide-recognizing CD4⁺ and CD4⁺CD45RA⁺ T-cell lines

Blood was collected from the Etablissement Français du Sang (EFS, Rennes, France) from anonymous healthy donors (these will be named 'donors' in the manuscript) after informed consent.

BP-HSA and BP-haptenated peptides specific CD4⁺ and CD4⁺CD45RA⁺ T-cell lines were generated as previously described¹⁸. CD4⁺ T-cells were restimulated on days 7, 14 and 21, with fresh autologous DCs loaded with BP-HSA¹⁸. For BP-haptenated peptide-specific T-cell stimulation, a pool of 6 peptides, each peptide being at a concentration of 10 µg/ml, were loaded on mature DCs for 4h. The same restimulation protocol as BP-HSA was then used.

Specificity of the CD4⁺ T-cell lines for BP-HSA or for the pool of BP-haptenated peptides was investigated using IFN-γ ELISpot assay on day 28 after 16 h of co-culturing CD4⁺ T-cells and DCs loaded with BP-HSA or peripheral blood mononuclear cells (PBMCs) loaded with a pool of BP-haptenated peptides respectively. The followings were used as controls: unloaded DCs and DCs loaded with HSA in the case of BP-HSA specific T-cell lines investigation, and unloaded PBMCs and PBMCs loaded with the pool of non-haptenated peptides for the pool of BP-haptenated peptides specific T-cell lines investigation. For BP-haptenated peptides, a second consecutive IFN-γ ELISpot assay was performed on day 29 with individual BP-haptenated peptides. Non-haptenated peptides were used as negative control. T-cell lines were considered BP-HSA specific when the production of

spots 2-fold higher in the presence of BP-HSA compared with cells loaded with HSA alone or without the protein, with a minimal difference of 30 spots. In the case of BP-peptides, a positive value was also assigned to culture wells with a spot count that was 2-fold higher in the presence of the BP-peptides compared with unmodified-peptides and cells alone, with a minimal difference of 30 spots.

T-cell stimulation with KLH (Keyhole Limpet Hemocyanin; Thermo Fisher Scientific) was used as a positive control¹⁸.

Anti-human HLA class II antibodies (anti-human HLA-DP, DQ and DR α blocking antibodies, kindly gifted by Dr. Bernard Maillère) were used to determine HLA molecules role in BP-haptenated peptide-specific T-cell responses. Antigen presenting cells were pre-treated with the different HLA class II subclasses antibodies prior to addition of BP-haptenated peptides and the T-cell lines.

Measurement of IgE antibodies specific to benzylpenicillin

Sera from the healthy donors were aliquoted and stored at -80° C. Specific IgE directed against BP was quantified using a fluorescence enzyme immunoassay (ImmunoCAP, Thermo Fisher Scientific, Uppsala, Sweden) as previously described²³. Limit of quantification for IgE was 0.1 kU/L. IgE levels of donors are in supplementary data S2.

HLA-DR genotyping of healthy donors

PBMCs from healthy donors were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma-Aldrich), and DNA was extracted using the NucleoSpin Blood L kit (Macherey-Nagel, Düren, Germany) as described by the manufacturer protocol. HLA-DR genotyping was performed using the All Set Gold SSP DRB1 typing kit (Invitrogen, Cergy, France), according to the manufacturer protocol. The HLA-DR genotype of donors is shown in supplementary data S2.

Benzylpenicillin-haptenated peptides HLA-class II binding ELISA assay

Binding to HLA-DR molecules was assessed by a competitive ELISA as previously described²⁴. The competition for the immunopurified HLA class II molecules was performed between a reporter peptide (biotinylated) and the peptide to be tested²⁴. Unlabeled forms of the reporter peptides were used as reference peptides to assess the quality of each experiment. The relative binding affinity reported was the ratio of the IC50 of the peptide to the IC50 of the reference peptide.

Lymphocyte transformation test

PBMCs from subjects with a history of allergy to penicillins and *in vivo* screened by skin prick tests were isolated by Lymphoprep (Oslo, Norway). 2×10^5 cells were cultured in triplicates for 6 days in antibiotic-free VLE RPMI 1640 medium (Biochrom GmbH, Berlin, Germany), supplemented with LPS-free 2×10^{-5} M 2-ME, 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate (complete medium; all from Sigma), and 5% autologous serum in 96 round-bottom microwell plates (Nunc; Nunc, Roskilde, Denmark) during 6 days²⁵ without or with different concentrations of either BP (0.5 – 1 – 2 mM), BP-HSA (10 – 50 – 100 μ M), HSA (10 – 50 – 100 μ M), individual BP-haptenated peptides and the corresponding non-haptenated peptides at 5 μ M. PHA and streptokinase (SK) were used as positive controls. Sixteen hours before harvesting, 0.5 μ Ci [³H]TdR (Perkin-Elmer, USA) was added to each well, and radionuclide uptake was measured by scintillation counting (Tomtec; Wallac, Turku, Finland). The stimulation index (SI) was calculated either as the ratio between the mean values of cpm obtained in stimulated cultures with BP and those obtained in the presence of medium alone or as the mean values of cpm obtained with BP-HSA and those obtained in the presence of HSA control or as the mean obtained in the presence of BP-haptenated peptides and those obtained in the presence of corresponding non-haptenated peptides control. LTT was considered positive when the SI was ≥ 3 at one concentration or ≥ 2 for a minimum of two tested concentrations.

Statistics

CD4⁺ T-cells frequency was estimated using the Poisson distribution according to the following formula:

$$\text{Frequency} = \frac{-\ln(\text{Number of negative T-cell lines} / \text{Total number of T-cell lines tested})}{\text{Number of CD4}^+ \text{ T-cells per well}}$$

To calculate statistical significance between stimulated and control conditions in allergic patients, triplicate values were compared between groups using a paired t-test. Tests were performed using GraphPad Prism software® (version 5.0, year, La Jolla, CA). Statistical tests (two-tailed) giving a p-value smaller than 0.05 were considered statistically significant.

Results

Detection and quantification of the frequency of blood-circulating CD4⁺ T-cells recognizing benzylpenicillin-human serum albumin conjugates

The overall results showed that CD4⁺ T-cells recognizing specifically BP-HSA were detected for all the tested donors, excepted donor 982, who was the only non-responding donor in our study. For all the tested donors we calculated an estimated frequency of pre-existing antigen-specific CD4⁺ T-cells in the blood, as previously described^{18,26,27}. Frequency of CD4⁺ T-cells recognizing BP-HSA varied depending on the donor from 0 to 0.74 BP-HSA-specific CD4⁺ T-cells per million of CD4⁺ T-cells with a mean value of 0.3 (Fig. 1A). The frequency for KLH specific CD4⁺ T-cells used as a positive control was 13.23 (data not shown).

We then assessed whether BP-HSA recognizing CD4⁺ T-cells involved in the immune response were naïve, as previously shown¹⁸. Naïve CD4⁺ T-cells characterized by the expression of CD45RA were isolated from the PBMCs of eight donors. BP-HSA CD4⁺ CD45RA⁺ and KLH CD4⁺ CD45RA⁺ T-cell lines were generated using the same methodology as described above. Results from the eight tested donors with BP-HSA showed that CD4⁺ CD45RA⁺ T-cell lines specific for BP-HSA were detected in seven donors among the eight tested (Fig. 1B). Only one donor (number 966) was negative. However, this donor showed only one positive T-cell line when using total CD4⁺ T cells. This discrepancy was probably due to the variability existing between the two experiments considering the low number of cell lines. Frequency of BP-HSA-specific naïve CD4⁺ T-cells was then calculated for each donor tested using the Poisson distribution (Fig. 1B). The calculated frequency varied from 0 to 2.23 naïve CD4⁺ T-cells recognizing BP-HSA per million of naïve CD4⁺ T-cells, corresponding to a mean value of 0.7.

CD4⁺ T-cell response to benzylpenicillin-haptenated peptides from human serum albumin

We then investigated the capacity of twelve selected peptides (see Scornet et al, 2016) from HSA and haptenated with BP to prime specifically CD4⁺ T-cells from different HLA-typed donors. T-cell lines generated from five donors are shown as representative results of the fourteen studied donors. Briefly, CD4⁺ T-cell lines from one donor were either able to respond to more than one BP-haptenated peptide (PM15-L3; PM16-L38; PM28-L2) or to only one BP-haptenated peptide (PM15-L14; PM16-L22; PM19-L13; PM43-L93; PM43-L15) (Fig. 2).

Five peptides were common to multiple donors (peptide LF-BP in 4 out of 13 tested donors; peptide PT-BP, 11/14; peptide FS-BP, 6/14; peptide VL-BP, 4/13; and peptide KV-BP, 5/14). Other peptides showed stimulating activity in only one or two donors (SF-2-BP; TG-BP and VC-BP). Four other peptides did not show any stimulating activity (LK-BP; LK-2-BP; SF-BP and LK-3-BP). Overall, the CD4⁺ T-cell response to BP-haptenated peptides involved mainly peptides PT-BP, FS-BP and KV-BP (Table 1; supplementary data S3). These three peptides had the BP bound on lysines 159, 212 and 525 from HSA respectively (Table 1).

Finally, we assessed the dose-response of PT-BP using specific T-cell lines isolated from two different donors. T-cell lines responded to a peptide concentration of less 0.1 $\mu\text{g/ml}$ for one donor and less 0.01 $\mu\text{g/ml}$ for the second donor (Fig. 3).

Role of HLA class II in the T-cell response to benzylpenicillin-haptenated peptides

To determine if the T-cell response to BP-haptenated peptides was mediated by HLA class II, we tested the capacity of antibodies specific for HLA-DR α , HLA-DP, and HLA-DQ molecules to inhibit the activation of BP-haptenated peptide-specific T-cell lines when stimulated with BP-haptenated peptides. In total 15 T-cell lines from three donors were tested with six BP-haptenated peptides (supplementary data S4). Our results showed that T-cell responses to BP-haptenated peptides were dependent on more than one HLA class II molecule.

***In vitro* CD4⁺ T-cell response to benzylpenicillin-human serum albumin conjugates supported by multiple CD4⁺ T-cell epitopes**

To identify CD4⁺ T-cell epitopes from BP-HSA, we derived CD4⁺ T-cell lines specific for BP-HSA from two healthy donors with different HLA-DR alleles using a T-cell assay as described previously²⁸. Purified CD4⁺ T-cells were seeded in multiple wells and stimulated weekly by autologous DCs previously loaded with BP-HSA to enrich the co-cultures in Ag-specific CD4⁺ T-cells. After three rounds of stimulation, each independent T-cell line (CD4⁺ T-cells present in a single well) was evaluated for its specificity using IFN- γ ELISpot. The fine specificity of the BP-HSA specific T-cell lines for BP-haptenated peptides was then evaluated in a second ELISpot assay using four BP-haptenated peptides to identify the haptenated peptides recognized by BP-HSA specific T-cell lines. As an example, the T-cell line number 24 from donor PM65 was specific for peptides FS-BP and KV-BP, whereas the T-cell line number 9 from the second donor PM66 was specific only for one peptide, namely PT-BP (Fig. 4A). On the other hand, we also investigated the specificity of BP-haptenated peptide induced T-cell lines from a single donor towards BP-HSA (or HSA, as control). Four PT-BP specific T-cell lines were reactive against BP-HSA (Fig. 4B).

Relative binding affinity of benzylpenicillin-haptenated and non-haptenated-peptides to HLA class II molecules

We then evaluated the capacity of these peptides to bind to ten HLA-DR molecules that are common alleles in the Caucasian population. We found that non-haptenated peptides LF and FS bound to seven different HLA-DR molecules. Peptide PT also exhibited a broad specificity for HLA-DR molecules by binding strongly to six alleles. Peptide SF bound three different HLA-DR molecules and KV only one allele. When peptides were haptenated with BP, only the LF peptide retained the same binding affinity. Conversely, peptides PT, SF and KV lost their binding affinity for some HLA molecules, acquiring a more efficient binding to new HLA molecules. Finally, the FS haptenated peptide lost

completely its binding affinity to most alleles only conserving binding to DR1 (supplementary data S5).

Haptenation position was also relevant. Haptenation of peptide LF on a central position (ex: the lysine in the pocket number 3 of the HLA-DRB4*0101 molecule) produces a two-log loss of the relative binding affinity. On the other hand, haptenation of the same peptide on a peripheral position (ex: the lysine in the pocket number -1 of the HLA-DRB3*0101 molecule and the lysine in the flanking sequence of the peptide for HLA-DRB5*0101 molecule) enhanced the relative binding affinity by two logs. The same observation was also true for peptides PT with three HLA alleles (DR15, DRB4 and DRB5), SF with one HLA allele (DRB4) and FS for several alleles (DR7, DR11, DR4, DR9, DRB4 and DRB5). Haptenation on a central position did not affect the binding affinity only in the case of peptide PT with the lysine in position number 3 of the DRB3 allele, and peptide SF with the lysine in position number 1 of the DRB4 allele. Haptenization of peptides on the 9 central pockets of the HLA binding groove seems to alter their conformation affecting their affinity to HLA molecules at least as evaluated with this method (supplementary data S5).

Benzylpenicillin-haptenated peptides and benzylpenicillin-haptenated human serum albumin stimulated PBMCs from penicillin allergic patients

We then asked whether BP-HSA and BP-haptenated peptides were able to directly stimulate PBMCs from patients allergic to penicillin derivatives. To this end, circulating mononuclear cells from 7 allergic patients were stimulated *in vitro* with either free BP, BP-HSA, the pool of the three BP-haptenated peptides that showed the highest frequencies of positivity using naïve T-lymphocytes from healthy donors (PT-BP; FS-BP; KV-BP) and each BP-haptenated peptides individually (with their respective controls). Three patients had a history of allergy to BP and four to AX-CLV (supplementary data S6). The results are showing that 6/7 of the patients responded to the BP-HSA bioconjugate. Of those, 5/6 also exhibited a positive proliferative response to, at least, one BP-haptenated peptide (Table 2; supplementary data S7). In particular, peptide PT-BP induced proliferation in patients MA and GS, and peptide KV-BP in patients MA and MM. Peptide FS-BP was not recognized by the T-cells from any of the allergic patients. PBMC from patient OA did not proliferate with BP-HSA nor with the pool of haptenated-BP peptides. Moreover, free BP was able to induce cell proliferation in patients MA and MM known to have a previous history of severe reaction to BP, but not in patients that showed adverse reaction to AX-CLV.

Discussion

Drug allergy constitutes an important health problem and among the molecules frequently involved penicillins are the most prevalent. Despite excellent research aiming at understanding the mechanisms of penicillin allergy, little is known about the process of patient immunization to penicillin-protein conjugates. Indeed, according to the hapten hypothesis small chemical molecules need to bind to proteins to be immunogenic. The objective of this work was to identify peptides from human serum albumin haptenated with BP and involved in naïve T-cells activation, immunization of patients and possibly related to clinical manifestations.

We first evaluated the capacity of a BP-HSA bioconjugate to prime CD4⁺ T-cells *in vitro* from sixteen healthy donors with different HLA genotypes to refine our previous evaluation of the frequency of pre-existing T-cells recognizing BP-HSA in healthy individuals¹⁸. By this approach, we estimated that there was 0.3 pre-existing CD4⁺ T-cell recognizing BP-HSA per million of CD4⁺ T-cells in the blood of healthy donors. Only one donor over the sixteen tested did not mount a BP-HSA-specific T-cell response. This frequency was in the range of what can be observed for therapeutic proteins²⁹.

In a companion work, our group identified BP binding sites on HSA at basic and physiological pH. Among the 59 lysines residues present in the HSA sequence, 51 were found to be occupied by BP when the reaction was performed at basic pH. However, only 21 binding sites were identified at physiological pH: Lys 12, 73, **137, 159**, 162, **190, 195, 199, 212, 351**, 402, 414, **432, 436**, 439, 475, 500, 519, **525, 541, 545**¹⁷. Twelve of these lysines (in bold) were identical to those identified by several groups in allergic patients sera and in bioconjugates synthesized *in vitro* with different penicillins^{15, 30-33}. Moreover, lysines 190, 199, 212 and 541 were found to be the most reactive residues on HSA reacting with β -lactams at low concentration and following a short incubation time^{12, 13-15, 30}. Based on these findings and using the IEDB-AR prediction approach, twelve peptides of 15-mers from HSA were selected and synthesized using a flexible approach by introducing an original BP-lysine synthon to the amino acid sequence of the peptide allowing a controlled BP haptenation on lysines of interest¹⁷. Selection of BP-haptenated peptides using the IEDB-AR was based on peptide/MHC affinity and the location of BP-haptenated lysine in the MHC binding grooves of the 7 most frequent HLA-DRB1 alleles from the Caucasian population.

We then evaluated the capacity of these selected BP-haptenated peptides to prime CD4⁺ T-cells *in vitro* from fourteen healthy individuals with different HLA genotypes and we were able to identify BP-peptides implicated in T-cell response to BP-HSA. Peptide PT-BP (Lys 159) was preferentially recognized by the vast majority of the donors. Meng *et al.* have identified Lysine 159 as a position occupied by BP only in BP-HSA bioconjugates synthesized *in vitro* and on HSA purified from patients' sera, however with a relatively lower frequency compared to other lysines¹⁵. Four other peptides presented high frequencies of positivity namely, LF-BP (Lys 137), FS-BP (Lys 212), VL-BP (Lys 351) and KV-BP (Lys 525). Lysine 525 and Lysine 212 have been described to be preferentially bound by benzylpenicillenic acid and flucloxacillin respectively^{15, 30}. Three other peptides gave rise to a lower frequency of T-cell responses namely SF-2-BP (Lys 199), TG-BP (Lys 432) and VC-BP (Lys 436). Four other peptides containing the lysine residues 190, 195 and 199 (LK-BP, LK-2-BP, SF-BP and LK-3-BP) were never recognized by T-cells from healthy donors despite the high reactivity of these lysines in the presence of β -lactam molecules^{12, 13, 15, 30}. Indeed, Lysine 190 has been found to be a primary target for modification with several drugs such as piperacillin, meropenem, aztreonam¹², BP¹⁵, flucloxacillin³⁰ and AX¹³. These results suggested that the biological response towards BP-peptides was not correlated with the chemical reactivity of the lysine positions. Two peptides, SF-2-BP (Lys 199) and SF-BP (Lys 195) having a common amino acid sequence but with BP occupying two different lysine position were differently recognized in tested donors. Peptide SF-2-BP was recognized by cells from two donors over the fourteen tested while peptide SF-BP was negative in all the tested donors. This observation strongly suggested that the lysine's position in the HLA binding groove was a primary requisite for T-cell recognition. Indeed, three peptides among the 14 tested, PT-BP, KV-BP, FS-BP, showed the highest frequency of positivity in the tested donors with BP bound on lysines 159, 525 and 212 respectively. These peptides were correctly identified using the IEDB *in silico* approach with BP facing the TCR and not facing the HLA class II molecule. Thus, we will refer these peptides as immunodominant³⁴⁻³⁶.

We then tested whether these three immunodominant BP-haptenated peptides from HSA were also recognized by T-cells from allergic patients. Patients were selected on their basis of clinical manifestations to amoxicillin or BP and reactivity in the LTT assay. In this latter case, LTT positivity could be in response to BP or AX conjugated or not to HSA. In our study, five allergic patients responded to both BP-HSA bioconjugate and to one or two BP-peptides, namely PT-BP (Lys 159) and KV-BP (Lys 525). These results confirmed that peptides PT-BP and KV-BP were implicated in the immunization process of allergic patients as previously found with normal donors and lysines 159 and 525 were important sites also in this context. Moreover, these results showed that memory T-cells from allergic patients were able to proliferate in response to the selected peptides indeed recognizing a BP-binding amino acid sequences similar to those derived from the processed BP-HSA bioconjugate and in agreement with the hapten hypothesis. Finally, as long as memory T-cells equally recognized both free BP as well as BP-HSA, this suggested that BP recognition could be dependent on both hapten and P-i concept, although direct binding of BP to HLA-bound peptide cannot be excluded.

In conclusion, we found that a large repertoire of naïve CD4⁺ T-cells specific for BP-HSA and BP bound to HSA-derived peptides was present in healthy donors. This study also identifies two peptides located on lysines 159 and 525 likely implicated in the immunization of allergic patients to penicillins. Recently, Meng *et al.* also published work using piperacillin and showing that the level of adducts formed in the circulation of patients was not different from the one required to activate T-cells using a Lys⁵⁴¹ peptide³⁷. These results suggested that a threshold level of drug–protein adduct was exceeded in all patients exposed to a therapeutic drug course and a large proportion of patients were at risk to be “immunized” to beta-lactams. Since our results obtained using cells from naïve subjects suggested that most individuals have a T cell repertoire that can be activated with BP-haptenated peptides, we believe that the propensity to be “immunized” and then to develop hypersensitivity may be due to an imbalance in the immune regulation. Concerning, the possibility of an “immunized” patient to develop hypersensitivity we can hypothesized that the imbalance between specific effector and regulatory T-cells is needed in addition to the homing of these effector cells to target organs. More work is needed to identify if putative epitope that could drive a Treg response do exist (T-regitopes) or if the T-effector/Treg response is mainly driven by the DC microenvironment through differential regulation of co-stimulatory/co-inhibitory signals^{38,39}.

Acknowledgments

We thank Dr. Francesca Brugnolo (Servizio Immunotrasfusionale e Terapie Cellulari, Azienda Ospedaliera Universitaria Pediatrica A. Meyer, Firenze) for providing blood samples from allergic patients and Mrs. Natacha Szely-Kerzerho for excellent technical support for HLA-DR genotyping. This project was supported by Biologie Servier, France.

Conflicts of Interest Statement

The authors declare no conflicts of interests.

Authors Contributions

MA performed experiments on healthy and allergic donors, analyzed the data and wrote the manuscript. RB performed experiments on healthy donors, reviewed and corrected the manuscript. NS and DJ performed the chemistry part of this study (monomer and peptide synthesis). LdC performed measurement of IgE antibodies specific to benzylpenicillin. LF and PP recruited allergic patients and performed experiments. BM designed the study and the HLA-class II binding ELISA assay. RW and NC took part in study conception. MP designed and supervised the conduct of the study and participated in data analysis and interpretation. MP, BM, DJ, and PP critically reviewed the manuscript and all authors approved the final version

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Table legends

Table 1: Frequency of CD4⁺ T-cell specific to benzylpenicillin-haptenated peptides in healthy donors.

Results were expressed as the number of responding donors to the 12 selected BP-haptenated peptides from HSA and the frequency of T-cells lines specific for BP-haptenated peptides among the total number of T-cell lines tested for each donor. HSA: Human Serum Albumin.

Table 2: PBMCs from allergic patients proliferate in response to benzylpenicillin-human serum albumin and benzylpenicillin-haptenated peptides.

PBMCs were cultured in the presence of free BP, BP-HSA bioconjugate, BP-haptenated peptides and their respective controls (untreated, HSA alone and non-haptenated peptides). Proliferation was measured by the addition of [3H]TdR for the final 16h of the experiment. Results correspond to the ratio between the mean values of cpm obtained in stimulated conditions and those obtained in the corresponding control as described in the Method section. LTT was considered as positive when the SI was ≥ 3 or ≥ 2 for at least two tested concentrations. BP, benzylpenicillin; SK, Streptokinase; PHA, Phytohemagglutinin.

Figure legends

Figure 1: Frequency of human CD4⁺ T lymphocytes recognizing benzylpenicillin-human serum albumin in donors.

Frequency of T-cells specific for BP-HSA was calculated for each donor using the Poisson distribution. For each donor, the number of T-cell lines specific for the antigen, out of the total number of T-cell lines tested is indicated in brackets. (A) Frequency of CD4⁺ T lymphocytes specific for BP-HSA (n=16). (B) Frequency of CD4⁺CD45RA⁺ T-cells specific for BP-HSA (n=8).

Figure 2: Specificity and magnitude of CD4⁺ T-cell responses against benzylpenicillin-haptenated peptides from human serum albumin in healthy donors.

CD4⁺ T-cell specificity for the individual peptide was assessed in two subsequent IFN- γ ELISpot assays. CD4⁺ T-cell lines were considered specific when a spot count was 2-fold higher in the presence of the BP-haptenated peptide compared to non-haptenated peptide. Data shows the number of IFN- γ spots from eight CD4⁺ T-cell lines specific for BP-haptenated peptides from HSA (n=5 healthy donors).

Figure 3: Dose-response of peptide-specific T-cell lines from healthy donors.

Peptide-specific T-cell lines from donors PM16 and PM19, were cultured with autologous DCs loaded with non-haptenated control peptides (10 µg/ml) or with a dose-range of the appropriate BP-haptenated peptides. The response was measured using IFN-γ ELISpot assay as described in the Methods section.

Figure 4: Cross-reactivity between benzylpenicillin-human serum albumin and benzylpenicillin-haptenated peptides-specific T-cell lines from donors.

(A) Positive CD4⁺ T-cell lines for BP-HSA were stimulated with autologous DCs loaded with BP-haptenated peptides or non-haptenated negative control peptides. T-cell line specificity was screened using an IFN-γ ELISpot assay as described in the methods section. (B) Positive CD4⁺ T-cell lines for a BP-haptenated peptide were stimulated with autologous DCs loaded with HSA or BP-HSA.

Table 1

			Total CD4 ⁺ T-cells										Naïve T-cells				
Position on HSA	Peptide annotation	Number of Responders	PM11	PM13	PM15	PM16	PM19	PM28	PM33	PM34	PM43	PM45	PM48	PM49	PM55	PM56	
K137	LF-BP	4/13	1/60				1/60				1/112		1/175				
K159	PT-BP	11/14	1/45		9/60	9/45	4/56			1/60	3/112	6/110	1/102	3/175	2/40	1/70	
K190	LK-BP	0/11															
K195	LK-2-BP	0/11															
	SF-BP	0/11															
K199	LK-3-BP	0/11															
	SF-2-BP	2/11	1/60								1/112						
K212	FS-BP	6/14	1/45	1/40	1/60				3/60		1/60		4/40				
K351	VL-BP	4/13	2/45								2/60	2/112	1/70				
K432	TG-BP	2/13					1/60				1/112						
K436	VC-BP	1/13	1/60														
K525	KV-BP	5/14	2/45	4/45				1/60		1/60				1/175			

Table 2

Patient	BP (mM)			BP-HSA (μ M)			Peptides (5 μ M)				SK 2500U/ml	PHA 1% vol/vol
	0.5	1	2	10	50	100	Pool of peptide- BP	PT-BP	FS-BP	KV- BP		
OA	0.7	0.5	0.6	0.7	0.5	0.6	1.1	NT	NT	NT	2.7	14.5
Z.S	2.2	1.1	1.1	0.9	3.8	9.3	3.5	NT	NT	NT	143.3	98.4
ME	0.8	0.8	1	1.2	3.3	5.1	4.1	NT	NT	NT	9.5	145.6
MA	4.8	10.2	16.1	2.3	2.1	4.7	NT	7.6	0.7	3.1	25.8	9.8
GS	0.8	1.1	1.1	0.5	7.7	1.5	NT	4.0	1.8	0.9	34.1	48.0
MM	11.2	19.2	21.5	2.2	2.1	0.6	NT	0.9	0.9	3.2	48.4	51.4
TD	0.9	0.5	0.4	1.4	2.3	4.6	NT	2.5	2.1	2.9	17.2	12.4

Figure 1

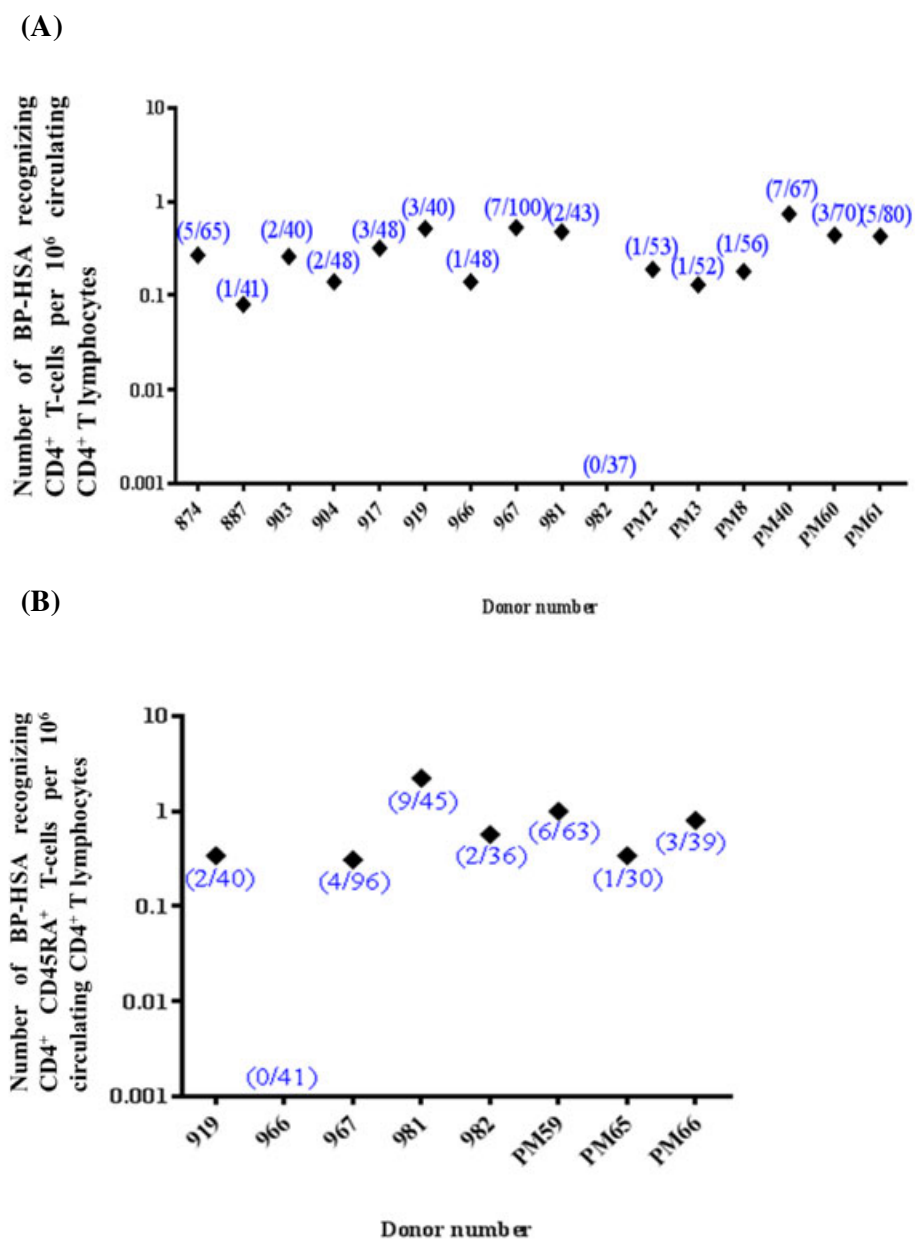


Figure 2

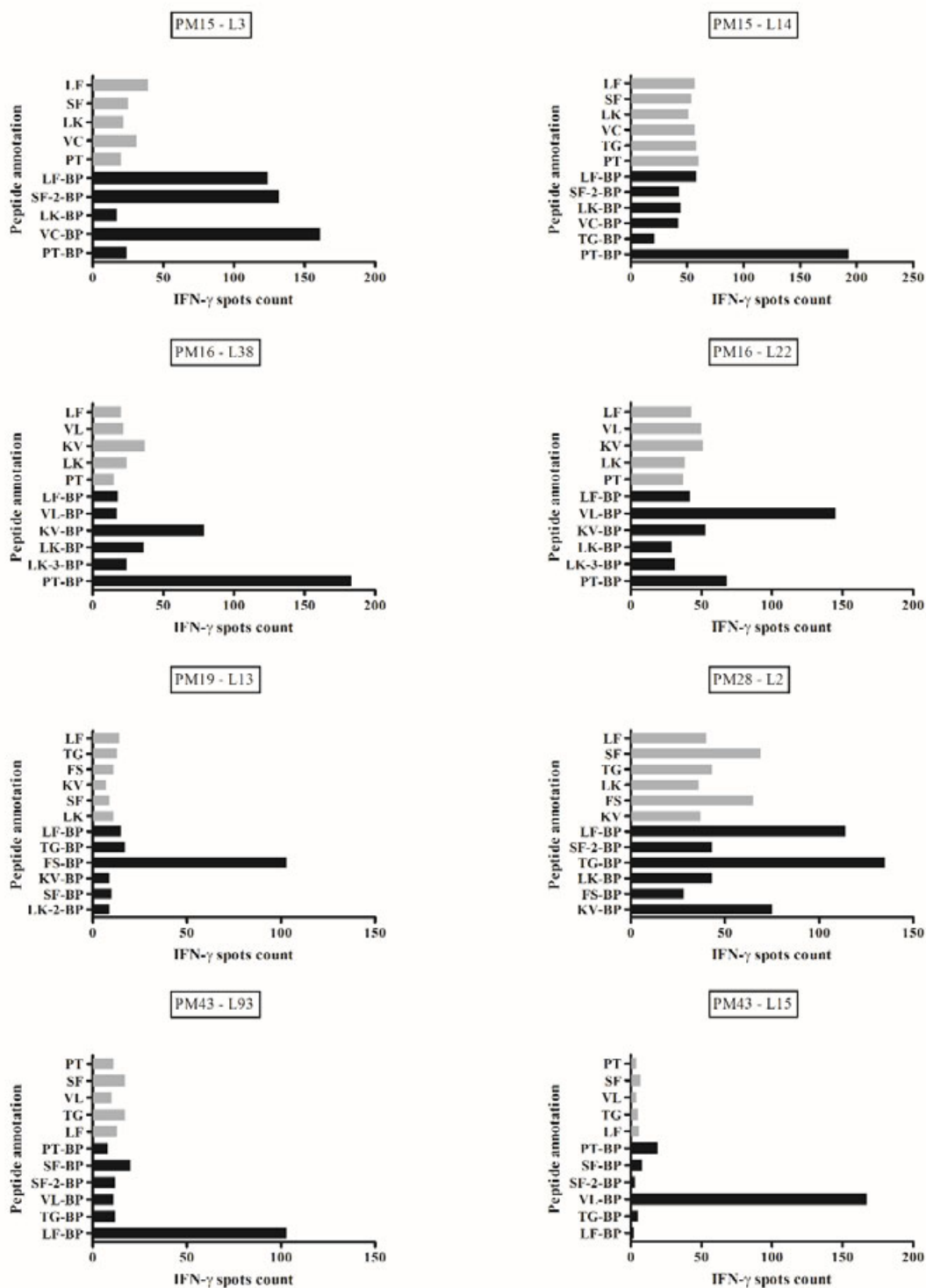


Figure 3

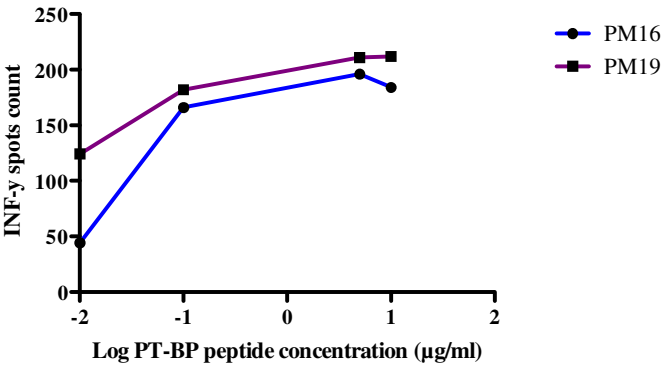
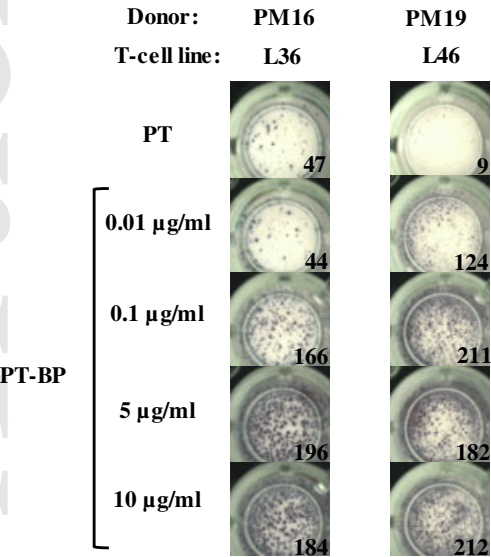
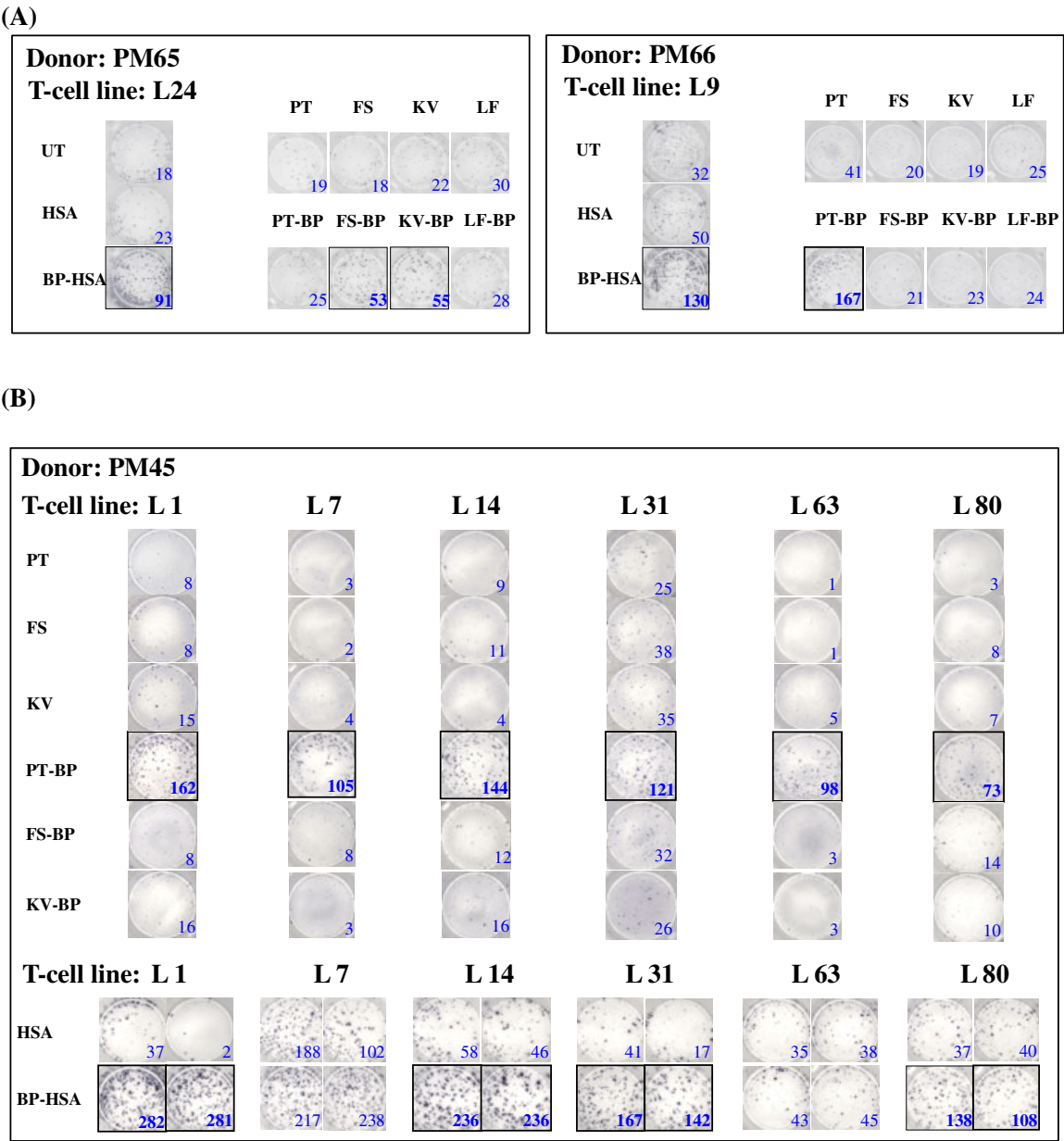


Figure 4



Supplementary data

S1: Benzylpenicillin-haptenated peptides sequences, annotations and lysine positions

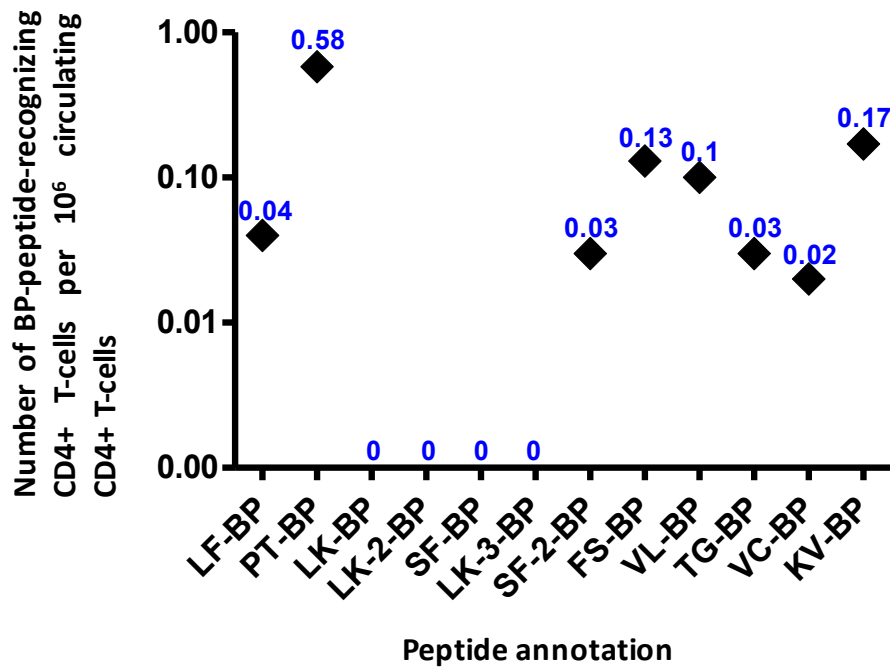
Position on HSA	Peptide sequence	Peptide annotation
K137	LKK*YLYEIARRHPYF	LF-BP
K159	PELLFFAK*RYKAAFT	PT-BP
K190	LRDEGK*ASSAKQRLK	LK-BP
K195	LRDEGKASSAK*QRLK SSAK*QRLKCASLQKF	LK-2-BP SF-BP
K199	LRDEGKASSAKQRLK* SSAKQRLK*CASLQKF	LK-3-BP SF-2-BP
K212	FGERAFK*AWAVARLS	FS-BP
K351	VVLLLRLLAK*TYETTL	VL-BP
K432	TPTLVEVSRNGLK*VG	TG-BP
K436	VEVSRNGLKVGSK*CC	VC-BP
K525	KERQIKK*QTALVELV	KV-BP

S2: HLA-DR genotype and IgE anti-benzylpenicillin determination of the studied donors.

HLA-DRB1 genotype was performed using the All Set Gold SSP DRB1 typing kit, and IgE specific to BP were measured in the sera of each donor using ImmunoCap Test as described in Methods section.

Donor numbers	HLA-DR genotyping	IgE (kU/L)
874	DRB1*01; DRB1*07; DRB4*01	< 0.1
887	DRB1*01; DRB1*11; DRB3*01	< 0.1
903	DRB1*13; DRB1*14; DRB3*01; DRB3*01	< 0.1
904	DRB1*03; DRB1*10; DRB3*01	< 0.1
917	DRB1*04; DRB1*15; DRB4*01; DRB5*01	< 0.1
919	DRB1*0701; DRB1*1303; DRB3*0101; DRB4*0101	< 0.1
966	DRB1*0301; DRB1*0407; DRB3*0101; DRB4*0101	< 0.1
967	DRB1*1501; DRB1*1601; DRB4*0101	< 0.1
981	DRB1*1101; DRB1*1501; DRB3*0202; DRB5*0101	< 0.1
982	DRB1*0301; DRB1*1302; DRB3*0101; DRB3*0201	< 0.1
PM2	DRB1*1101; DRB1*1401; DRB3*0202	< 0.1
PM3	DRB1*0101; DRB1*0101	< 0.1
PM8	DRB1*0102; DRB1*1501; DRB4*0101; DRB5*0101	< 0.1
PM11	DRB1*0401; DRB1*1303; DRB3*0101; DRB4*0101	< 0.1
PM13	DRB1*1101; DRB1*1501; DRB3*0109; DRB5*0101	< 0.1
PM15	DRB1*1301; DRB1*1401; DRB3*0101; DRB3*0101	< 0.1
PM16	DRB1*0404; DRB1*1502; DRB4*0101; DRB5*0101	< 0.1
PM19	DRB1*1301; DRB1*1401; DRB3*0109; DRB3*0202	< 0.1
PM28	DRB1*0901; DRB1*1001; DRB4*0101	< 0.1
PM33	DRB1*1101; DRB3*0202	< 0.1
PM34	DRB1*0101; DRB1*0102	< 0.1
PM40	DRB1*1101; DRB1*1401; DRB3*0202	< 0.1
PM43	DRB1*0102; DRB1*1101; DRB3*0202	< 0.1
PM45	DRB1*0701; DRB1*1301; DRB3*0201; DRB4*0101	< 0.1
PM48	DRB1*0804; DRB1*1101; DRB3*0101	< 0.1
PM49	DRB1*1302; DRB3*0202; DRB3*0201	< 0.1
PM55	DRB1*1302; DRB1*1602; DRB3*0201; DRB5*0101	< 0.1
PM56	DRB1*0405; DRB1*1301; DRB3*0202; DRB4*0101	< 0.1
PM59	DRB1*0301; DRB1*1302; DRB3*0101; DRB3*0201	< 0.1
PM60	DRB1*0801; DRB1*1101; DRB3*0101	< 0.1
PM61	DRB1*1301; DRB1*0701; DRB3*0201; DRB4*0101	< 0.1
PM65	DRB1*0102; DRB1*1501; DRB5*0101	< 0.1
PM66	DRB1*0301; DRB1*1302; DRB3*0101; DRB3*0201	< 0.1

S3: Frequency of human CD4+ T lymphocytes recognizing benzylpenicillin-haptenated peptides. Frequency of T-cells specific for BP-peptides was calculated for each donor using the Poisson distribution. And a mean frequency of T-cells repertoire for each BP-peptide is showed in this figure.



S4: T-cell response to benzylpenicillin-haptenated peptides was mediated by HLA class II molecules. BP-haptenated peptide specific T-cell lines ($1-3 \times 10^4$ cells) were incubated with mature DCs loaded with BP-haptenated peptides (10 µg/ml) in the presence of anti-HLA class II antibodies (L243 anti-HLA-DRα or SPVL3 anti-HLA-DQ or B7/21 anti-HLA-DP) added directly into the culture. T-cell activation was assessed by IFN-γ ELISpot assay. Results are expressed as percentage inhibition of T-cell activation, as assessed by IFN-gamma production, in the presence of anti-DR or anti-DP or anti-DQ compared to non-treated cells. All cell tested cell lines were treated with anti-DR, anti-DQ or anti-DP, only results showing a significant effect are shown.

Peptide annotation	Donor/ T cell line number	HLA locus	Percentage inhibition of T-cell activation (%)
FS-BP	PM34/L3	DQ	39
	PM34/L30	DRα	63
PT-BP	PM43/L36	DQ	76
	PM34/L20	DRα	64
	PM43/L47	DRα	73
	PM43/L76	DRα and DP	59.5 (DR) and 54.5 (DP)
	PM43/78	DRα	59.66
	PM45/L7	DRα	78.95
	PM45/L63	DRα and DP	90 (DR) and 71.5 (DP)
	PM45/L80	DRα	69
VL-BP	PM34/L20	DRα	57
	PM43/L92	DRα and DP	78.5 (DR) and 55 (DP)
	PM43/L107	DRα	76.5
LF-BP	PM43/L93	DP	66.5
TG-BP	PM43/L49	DRα	66
KV-BP	PM43/L23	DRα	51
	PM43/L107	DRα	76.5

S5: Binding affinities of benzylpenicillin-haptenated and non-haptenated peptides to HLA class II molecules. Binding affinity was evaluated using a competitive ELISA assay with HLA class II specific biotinylated reporter peptides and immunopurified HLA class II molecules as described in the Methods section. Unlabeled forms of the reporter peptides served as reference. Data reported are the ratio of the IC50 of the peptides to the IC50 of the reference peptide. Values in bold correspond to good binders (ratio inferior to 100). Underlined values correspond to a two log difference between the relative binding affinity of non-haptenated peptides and BP-haptenated peptides for the same HLA allele. Score for lysine position in the HLA binding groove is indicated by: -, located in the flanked region; **0**, located in a position oriented towards MHC molecule; **1**, located in a position oriented towards the TCR. In brackets are indicated the exact position of the lysine in the pocket. K, lysine.

Position on HSA	Peptide annotation	Parameter	DR7	DR3	DR1	DR11	DR15	DR4	DR9	DRB3	DRB4	DRB5
K137	LF	Relative Binding	36	4	3	0.2	228	2	1	<u>>4 090</u>	<u>0.3</u>	<u>183</u>
		K Position	1 (-1)	-	1(-1)	1 (-1)	1 (-1)	1 (-1)	1 (-1)	1 (-1)	1 (3)	-
	LF-BP	Relative Binding	3	5	0.5	0.3	186	5	8	<u>171</u>	<u>4 688</u>	<u>1</u>
K159	PT	Relative Binding	632	nd	1 357	0.1	8	64	49	<u>1 554</u>	<u>29</u>	<u>50</u>
		K Position	1 (2)	1 (5)	0 (4)	1 (5)	0 (6)	1 (5)	1 (2)	1 (3)	1 (8)	0 (6)
	PT-BP	Relative Binding	>1 064	11	2 097	7	<u>1 300</u>	250	641	<u>69</u>	<u>>4 090</u>	<u>4 474</u>
K195	SF	Relative Binding	139	71	48	70	800	>252	1 164	572	<u>1 500</u>	896
		K Position	-	-	1 (-1)	-	0 (1)	0 (1)	0 (1)	-	0 (1)	-
	SF-BP	Relative Binding	79	471	12	710	147	>252	625	421	<u>29</u>	2 146
K212	FS	Relative Binding	<u>6</u>	>1 176	6	<u>1</u>	380	<u>3</u>	<u>3</u>	2 067	<u>1</u>	<u>0.4</u>
		K Position	1 (2)	1 (3)	1 (5)	1 (2)	1 (2)	1 (3)	1 (5)	1 (2)	1 (5)	1 (3)
	FS-BP	Relative Binding	<u>>1 064</u>	>1 176	20	<u>>490</u>	>986	<u>>252</u>	<u>>8 333</u>	>644	<u>>4 090</u>	<u>>4 313</u>
K525	KV	Relative Binding	215	294	41	>490	620	304	635	>4 090	3 219	1 126
		K Position	1 (2)	1 (3)	1 (3)	1 (3)	0 (4)	1 (3)	1 (3)	0 (1)	0 (6)	1 (3)
	KV-BP	Relative Binding	>1 064	>1 176	265	290	35	>252	>8 333	>644	531	>4 313

S6: Clinical history of patients. AX, amoxicillin; BP, benzylpenicillin; CLV, clavulanic acid; F, Female; LTT, lymphocyte transformation test; M, Male; MDM, minor determinant mixture; PPL, benzylpenicilloyl-polylysine (major determinant); yo, years old; NT, not tested.

Patient	Age /Sex	Drug	Symptoms	Age of first reaction	Latency	Skin Prick Test (PPL/MDM/AX/Cef)	Intradermal test	Drug provocation test	Patch test	RAST β -lactams	LTT
OA	16/M	AX-CLV	Drug fever, exanthema	14 yo	Immediate	Negative	Negative	Positive	NT	Negative	Positive AX
Z.S	12/M	AX-CLV	Urticaria	6 yo	Delayed	Negative	Positive	NT	NT	Positive	Positive AX, HSA-AX and HSA-BP
ME	12/F	AX-CLV	Drug fever, exanthema	10 yo	3rd day of therapy	Negative	Negative	Positive	NT	Negative	Positive AX, HSA-AX and HSA-BP
MA	31/M	BP	Anaphylactic shock, angioedema	4 yo	Immediate	NT	NT	NT	NT	NT	Positive BP and HSA-BP
GS	24/F	AX-CLV	Urticaria, severe skin rash	4 yo	Immediate	NT	NT	NT	NT	NT	Positive HSA-BP and HSA-AX
MM	60/F	BP	Angioedema, dyspnoea, generalized pruritus	34 yo	Immediate	Positive at late reading	NT	NT	Positive BP	Positive	Positive BP, HSA-BP and AX
TD	36/M	BP	Urticaria, severe skin rash	11 yo	Immediate	NT	NT	NT	NT	NT	Positive HSA-BP

S7: PBMCs from allergic patients proliferate in response to benzylpenicillin-human serum albumin and benzylpenicillin-peptides. PBMCs were cultured in the presence of free BP, BP-HSA bioconjugate or BP-haptenated peptides. Proliferation was measured by the addition of [3H]TdR for the final 16h of the experiment. Results are shown as the mean cpm $\pm$ SD of triplicate cultures. *P<0.05.

