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Identification and frequency of circulating CD4⁺ T lymphocytes specific to Benzylpenicillin in healthy donors

C. Nhim¹, S. Delluc², F. Halgand³, L. de Chaisemartin^{1,4}, R. J. Weaver⁵, N. Claude⁵, D. Joseph⁶, B. Maillère⁷ & M. Pallardy¹

¹Université Paris-Sud, INSERM UMR 996, UFR Pharmacie, Châtenay-Malabry; ²Indicia Biotechnology, Oullins; ³Plate-forme Protéomique Biogenouest, IRSET, INSERM UMR 1085, Rennes; ⁴Unité Fonctionnelle d'Immunologie 'Autoimmunité et Hypersensibilités', AP-HP, Hôpital Bichat-Claude Bernard, Paris; ⁵Institut de Recherches Internationales, Suresnes; ⁶Université Paris-Sud, UMR CNRS 8076, UFR Pharmacie, Châtenay-Malabry; ⁷Service d'Ingénierie Moléculaire des Protéines, Institut de Biologie et de Technologies de Saclay, Commissariat à l'Energie Atomique et aux Energies Alternatives, Gif-sur-Yvette, France

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Correspondence

Marc Pallardy, PhD, Université Paris-Sud, INSERM UMR-S 996, UFR Pharmacie, 5 Rue Jean-Baptiste Clément, 92296 Châtenay-Malabry, France.
Tel.: +00-33-(0)1-46-83-54-92
Fax: +00-33-(0)1-46-83-54-96
E-mail: marc.pallardy@u-psud.fr

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Abstract

Background: Drug hypersensitivity is known to rely on a drug-specific T-cell response. Amplitude of antigen-specific T-cell response is partly controlled by the size of the antigen-specific naïve CD4⁺ T-cell repertoire, but estimate of this repertoire has never been investigated for allergenic drugs. The purpose of this study was to evaluate the frequency of benzylpenicillin-specific CD4⁺ T lymphocytes in healthy donors.

Methods: Co-cultures were established with CD4⁺ T lymphocytes from healthy donors and mature autologous dendritic cells loaded with benzylpenicillin coupled to human serum albumin. CD4⁺ T lymphocytes were stimulated once a week for 4 weeks with benzylpenicillin coupled to human serum albumin. The CD4⁺ T-cell response was measured using an interferon- γ ELISPOT assay. Frequency of benzylpenicillin-specific naïve CD4⁺ T lymphocytes was then calculated using the Poisson distribution law.

Results: Results showed the presence of benzylpenicillin-specific CD4⁺ T lymphocytes in 9 of 10 tested healthy donors irrespective of their HLA typing, with a mean frequency of 0.29 cells per million of CD4⁺ T cells. Experiments performed on naïve (CD45RA⁺) and on memory (CD45RO⁺) CD4⁺ T lymphocytes showed that these benzylpenicillin-specific CD4⁺ T lymphocytes belonged to the naïve T-cell subpopulation.

Conclusion: This study showed for the first time the existence of naïve CD4⁺ T lymphocytes specific to benzylpenicillin in healthy donors.

Benzylpenicillin (BP) is an antibiotic of the β -lactam family known to provoke allergic reactions in treated patients with a prevalence around 1.9% (1). Clinical manifestations of BP allergy include not only urticaria, angioedema, and bullous skin eruptions but also severe reactions like anaphylactic shock (2). Human T lymphocytes specific to BP have been isolated from peripheral blood of allergic patients and were able to

proliferate *in vitro* in response to BP stimulation, demonstrating their involvement in hypersensitivity reactions (3–7).

Benzylpenicillin is not immunogenic by itself and is considered as a hapten. Benzylpenicillin can bind covalently to the nucleophilic primary amine function of lysine residues of targeted proteins through the opening of the β -lactam ring forming bioconjugates (3, 7). Benzylpenicillin binding to human serum albumin (HSA) on different lysines have been found both *in vivo* using sera of allergic patients and *in vitro* by synthesizing bioconjugates (7–10). T cells from allergic patients were also found to react with HSA-BP, but the question remains how this T-cell specificity emerges with a selected number of synthetic molecules.

Abbreviations

BP, benzylpenicillin (or penicillin G); DCs, dendritic cells; HSA, human serum albumin; IFN- γ , interferon γ ; PBMCs, peripheral blood mononuclear cells.

The CD4⁺ T-cell repertoire is mainly formed by positive and negative selections in the thymus leading to the generation of functional CD4⁺ T cells able to recognize foreign peptides presented by HLA class II molecules (11). Due to the artificial nature of BP-T-cell epitopes, it is not clear whether selection of BP-specific T cells is a common phenomenon or remains limited to few donors. Selection of BP-specific T cells could be a relatively rare event accounting for the low occurrence of BP allergy. On the other hand, a large T-cell repertoire found in multiple donors would underline the potential of BP to be recognized by many donors.

To shed some lights on the shaping of BP-specific T-cell repertoire, we investigated the frequency of the CD4⁺ T lymphocytes specific to HSA-BP in healthy donors, by applying the cellular approach recently described to evaluate the CD4⁺ T-cell response to therapeutic proteins (12, 13). T lymphocytes were stimulated once a week with autologous dendritic cells (DCs) loaded with HSA-BP to amplify HSA-BP-specific T cells. Human serum albumin-benzylpenicillin-specific T cells were subsequently detected by interferon- γ (IFN- γ) ELISPOT, and their frequency at the initiation of the culture was calculated using the Poisson distribution law.

Methods

Synthesis of human serum albumin–benzylpenicillin bioconjugates

Human serum albumin-benzylpenicillin was produced at pH 7.4 and at pH 11 (basic pH). At neutral pH, HSA-BP was produced by mixing 100 mg of HSA (Sigma-Aldrich, St. Louis, USA) with 30 mg of BP (Sigma-Aldrich) in 10 ml of phosphate buffer (0.01 M; pH = 7.4; Invitrogen, Carlsbad, USA) and kept for 24 h, at 37°C. Human serum albumin-benzylpenicillin at basic pH was produced by mixing 100 mg of HSA with 229 mg of BP in 6 ml of carbonate buffer (0.1 M, pH = 11; Sigma) and kept for 18 h at 37°C. A negative control (HSA neutral pH and HSA basic pH) was produced using 100 mg of HSA in the same conditions, without BP. Unbound penicillin was removed by conducting a 5-day dialysis against distilled water using Thermo Scientific dialysis cassettes with a 10-kDa molecular weight cutoff or by conducting diafiltrations with ammonium acetate buffer using Thermo Scientific Pierce protein concentrators with a 20-kDa molecular weight cutoff. The absence of unbound penicillin was controlled by LC-MS analysis on a final methanol washing of the resulting dialyzed bioconjugates.

Detection of BP binding to HSA using Western blot

Twenty micrograms of denaturated proteins was loaded into a 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, Chalfont St Giles, UK). Benzylpenicillin was revealed with a mouse anti-penicillin monoclonal antibody (Serotec, Oxford, UK). Immunoreactive bands were detected by enhanced chemiluminescence (EZ-ECL detection kit; Clinisciences, Nanterre, France).

Global mass measurements using MALDI-MS

One micro litre of a 10 μ M sample solution diluted in a 50 mM ammonium acetate buffer (Sigma-Aldrich) was deposited on the MALDI target plate and mixed with 1 μ l of a 10 mg/ml sinapinic acid solution (Sigma-Aldrich) prepared in 70% acetonitrile solution (Sigma-Aldrich) containing 0.1% formic acid (Sigma-Aldrich). MALDI mass spectra were acquired in the linear mode on a Bruker MALDI-MS instrument (Ultraflex, BrukerDaltonik GmbH). Mass spectra were analyzed using the FlexAnalysis (version 3.0) software using the algorithm SNAP for peak detection. MALDI experiments were realized for both HSA-BP produced under basic condition and HSA-control treated under the same basic conditions in the absence of BP.

Fifty micro litre of a 10 μ M solution of HSA-BP and native HSA in ammonium carbonate buffer were reduced at 56°C by 10 μ l of a 65 mM DTT solution for 1 h and alkylated with 135 mM iodoacetamide in the dark at room temperature. The samples were then digested overnight at 37°C with 4 μ l of a solution of trypsin (Promega, Madison, WI) in a concentration of 0.1 μ g/ μ l. Finally, the samples were washed with 30 μ L of 90% acetonitrile solution containing 1% formic acid. The MS measurements were performed with a nanoflow high-performance liquid chromatography (HPLC) system (Dionex, LC Packings Ultimate 3000) connected to a hybrid LTQ-Orbitrap Velos spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (New Objective). Tryptic digests of penicilloylated HSA were analyzed by nano-LC-MS/MS using a high collision dissociation (HCD) mode.

Blood collection and measurement of antibodies specific to BP

Blood was collected from the Etablissement Français du Sang (EFS, Rungis, France) from anonymous healthy donors (these will be named 'donors' in the manuscript) after informed consent. Sera from these 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 described (14). Limit of quantification for IgE was 0.1 kU/l. Sera were also collected from ten volunteers with no documented allergy to penicillins and different from the donors, they were named the 'control group'.

HLA-DRB1 genotyping of healthy donors

Peripheral blood mononuclear cells 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 manufacturer's protocol. HLA-DRB1 genotyping was performed using the All Set Gold SSP DRB1 typing kit (Invitrogen), according to the manufacturer protocol.

Generation of HSA-BP-specific CD4⁺ and CD4⁺ CD45RA⁺ T-cell lines

Monocyte-derived dendritic cells (DCs) were generated from plastic-adherent PBMCs after 4 days of culture in AIM-V medium (Invitrogen) supplemented with human recombinant IL-4 and GM-CSF (R&D Systems, Minneapolis, USA). CD4⁺ T lymphocytes were isolated from PBMCs by positive selection using anti-CD4 monoclonal antibody coupled to magnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Naive CD4⁺ CD45RA⁺ and memory CD4⁺ CD45RO⁺ T lymphocytes were isolated from PBMCs by negative selection using specific microbeads (Miltenyi Biotech).

Percentage of CD4⁺, CD4⁺ CD45RA⁺, and CD4⁺ CD45RO⁺ positive cells was evaluated by flow cytometry. The following monoclonal antibodies were used: CD4-FITC, CD8-PE, CD45RA-PE, CD45RO-PE (Becton Dickinson, Franklin Lakes, USA), and appropriate isotype controls were also used to determine the nonspecific staining. Cells were analyzed using a FACSCalibur cell analyzer (Becton Dickinson).

For T-cell stimulation, DCs were loaded with 10 µM of HSA-BP or with KLH (Keyhole Limpet Hemocyanin; Thermo Fisher Scientific), as a positive control. After DC maturation using 1 µg/ml of LPS (Sigma-Aldrich) overnight, 10⁴–3 × 10⁴ loaded DCs were then added to 10⁵–3 × 10⁵ autologous CD4⁺ T lymphocytes per round-bottom micro-well containing 200 µl Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% human AB serum (Lonza, Basel, Switzerland), 1000 U/ml of rh-IL-6 and 10 ng/ml rh-IL-12 (R&D Systems). CD4⁺ T lymphocytes were restimulated on days 7, 14, and 21, with fresh autologous DCs loaded with KLH or HSA-BP and with 10 U/ml of rh-IL-2 and 5 ng/ml rh-IL-7 (R&D Systems). Specificity of the CD4⁺ T-cell lines was investigated using IFN-γ ELISPOT assay at day 27.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assay was performed as described previously after 16 h of co-culturing CD4⁺ T cells and DCs loaded with KLH or HSA-BP (12, 13). The following were used as controls: unloaded DCs or DCs loaded with native HSA or HSA treated at basic pH. Spots were revealed by the addition of extravidin conjugate and BCIP substrate (Sigma-Aldrich). Only T-cell lines with a spot count of 30 (minimum) were analyzed.

Statistics

CD4⁺ T cells' frequency was estimated using the Poisson distribution according to the following formula:

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

Table 1 HLA-DR genotype and anti-BP IgE antibodies of studied donors. HLA-DRB1 genotype was performed using the All Set Gold SSP DRB1 typing kit, from Invitrogen. IgE specific to BP was measured in the sera of each donor using the ImmunoCap Test from Thermo Fisher

Donor numbers	IgE (kU/l)	HLA-DRB1 genotyping
874	<0.1	DR01; DR07
887	<0.1	DR01; DR11
903	<0.1	DR13; DR14
904	<0.1	DR03; DR10
917	<0.1	DR04; DR15
919	<0.1	DR07; DR13
966	<0.1	DR03; DR04
967	<0.1	DR15; DR16
981	<0.1	DR11; DR15
982	<0.1	DR03; DR13
Control group (n = 10)	<0.1	ND
	<0.1	
	<0.1	
	<0.1	
	<0.1	
	<0.1	
	<0.1	
	<0.1	
	<0.1	
	<0.1	
	<0.1	

ND, Not determined.

Results

Donor characterization

IgE specific to BP was quantified to determine whether the studied donors were recently immunized to BP or not (Table 1). Moreover, we also tested ten additional volunteers, who did not experience any documented allergy to penicillins; these volunteers constituted the 'control group'. Results showed that no anti-BP-specific IgE could be detected in either the ten studied donors or in the control group. HLA-DRB1 genotyping was performed for these ten studied donors. Results showed that these donors harbored different HLA-DRB1 allotypes. The seven most frequent HLA-DRB1 allotypes of the Caucasian population were all present in our panel of donors (Table 1): HLA-DR01, DR03, DR04, DR07, DR11, DR13, and DR15.

Characterization of the HSA-BP bioconjugates

Western blotting experiments on HSA-BP using a mouse antipenicillin monoclonal antibody showed immunoreactive bands for BP-HSA produced at neutral and basic pH, revealing the presence of BP covalently bound to HSA (Fig. 1A). MALDI-TOF mass spectrometry experiments measured an

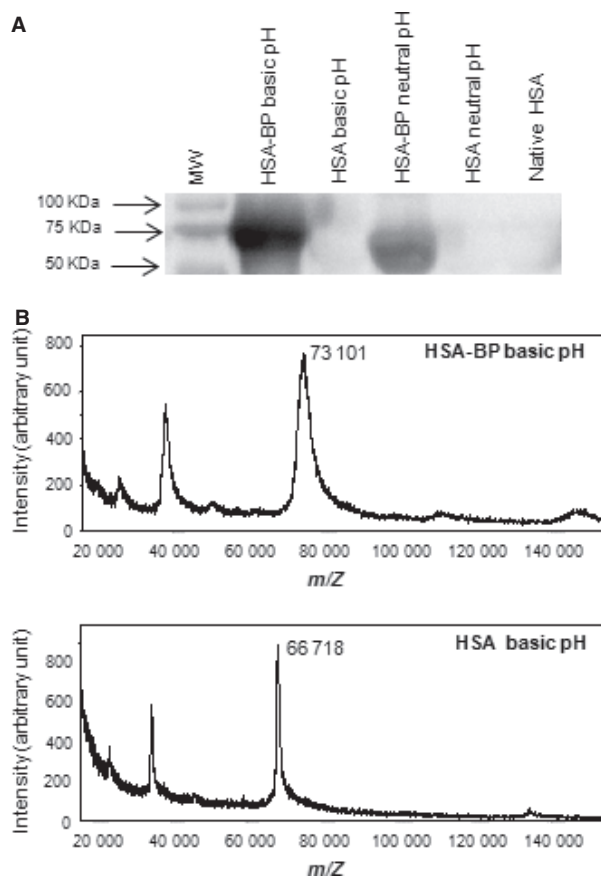


Figure 1 Characterization of HSA-BP bioconjugates. (A) Detection of BP binding to HSA in HSA-BP bioconjugates synthesized at basic and neutral pH by Western blot using a mouse antipenicillin monoclonal antibody (MW: molecular weight). (B) Mass spectrometry profile of HSA-BP bioconjugate synthesized at basic pH and HSA treated at basic pH.

experimental molecular mass of 73101 Da for HSA-BP produced at basic pH (Fig. 1B) compared with 66718 Da for native HSA treated at basic pH. This result suggested an average of nineteen BP molecules bound per HSA molecule (BP mw is 334.4 Da), and 17 lysine-penicilloylated tryptic peptides were clearly identified. However, MALDI-TOF analyses can only give an estimate on the number of penicilloylated sites.

Analyses of tryptic digests of both HSA-BP and native HSA by MALDI-MS experiments allowed the disappointing identification of only nine BP-modified peptides bearing 1–3 haptens in comparison with experimental parent ion masses of both samples to theoretical m/z values calculated for all possible peptides with one to several missed cleavage(s) at lysine residue(s) and added a multiple of 334 amu (mass of BP). Tryptic digests were analyzed by MALDI-MS and nano-LC-MS/MS in high collision dissociation (HCD) mode. The detection of penicilloylated sites was identified thanks to the two characteristic ion fragment masses of BP hapten (m/z 160.04 and 217.06) (7) correlated with the m/z values for

the selected tryptic BP-peptides bearing missed cleavages. Seventeen sites of penicilloylation over the average nineteen expected were detected.

Detection of BP-specific CD4⁺ T-cell lines

Benzylpenicillin CD4⁺ T-cell lines from donors were generated after three weekly repeated stimulations by autologous mature DCs loaded with HSA-BP. As culture conditions induced a Th1 polarization, IFN- γ ELISPOT assay was performed to detect CD4⁺ T-cell lines specific to HSA-BP. CD4⁺ T-cell line was considered positive when the number of spots obtained in the presence of DCs loaded with HSA-BP was at least twice the number of spots obtained with the controls (unloaded DCs, DCs loaded with native HSA and DCs loaded with HSA treated at basic pH). Results from donor 917 are shown as a representative result for the ten studied donors (Fig. 2). For this donor,

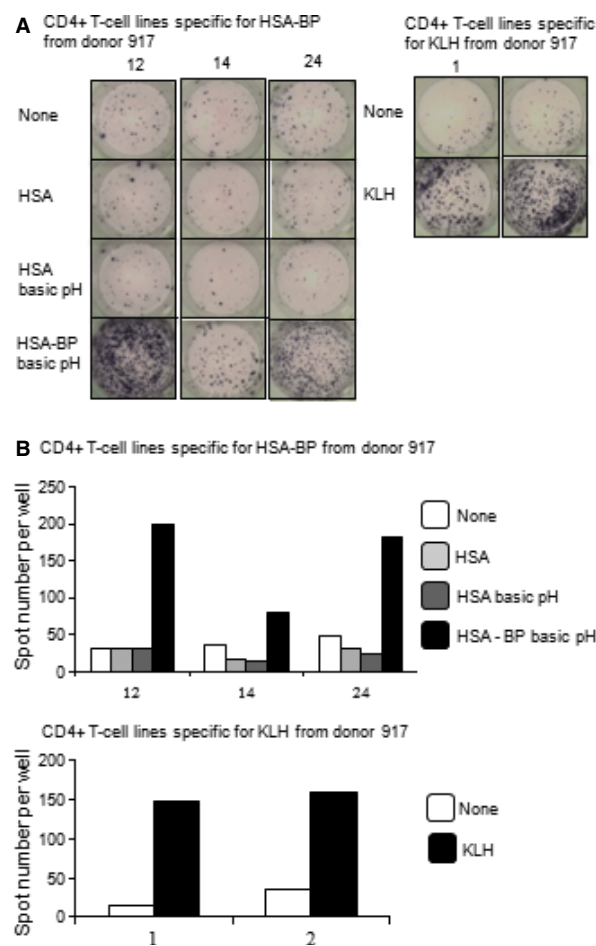


Figure 2 CD4⁺ T-cell lines specific to HSA-BP and KLH. (A) IFN- γ ELISPOT response of CD4⁺ T cells stimulated with unloaded DC (none) or DC loaded with either native HSA or HSA treated at basic pH (HSA basic pH) or HSA-BP bioconjugates (HSA-BP basic pH) or KLH. (B) Spot numbers for CD4⁺ T-cell lines specific to HSA-BP or KLH.

three T-cell lines (number 12, 14, and 24) were found positive for HSA-BP among the forty-eight T-cell lines tested. For all the donors tested, KLH T-cell lines were also generated as a positive control and were able to secrete specifically IFN- γ in the presence of autologous DC loaded with KLH (Fig. 2).

Quantification of the frequency of blood-circulating BP-specific CD4⁺ T lymphocytes

Although all the wells were cultivated under the same conditions, antigen specificity of the resulting T-cell lines greatly varied from one well to another. Preexisting BP-specific T cells are expected to be present in the blood of healthy donors at very low frequency and are therefore distributed in a heterogeneous manner at the initiation of the experiment. With our experimental settings, this distribution follows the Poisson distribution law based on the percentage of negative T-cell lines that do not contain preexisting BP-specific CD4⁺ T lymphocytes (Fig. 3). CD4⁺ T lymphocytes specific to BP were detected for all donors tested, excepted donor 982, who was the only nonresponding donor found in our study. Frequency of CD4⁺ T lymphocytes specific to BP varied depending on the donor from 0 to 0.53 BP-specific CD4⁺ T lymphocytes per million of CD4⁺ T lymphocytes with a mean value of 0.29.

BP-specific CD4⁺ T lymphocytes are from the naive compartment

We then tested whether BP-specific CD4⁺ T lymphocytes involved in the immune response were naive or memory T lymphocytes. Naive CD4⁺ T lymphocytes characterized by the expression of CD45RA were isolated from the PBMC of five of the ten healthy donors studied. Benzylpenicillin CD4⁺ CD45RA⁺ and KLH CD4⁺ CD45RA⁺ T-cell lines were

generated using the same methodology as described above. Results from the five donors tested with HSA-BP showed that CD4⁺ CD45RA⁺ T-cell lines specific to BP were detected in four donors among the five tested (Fig. 4). Only one donor (number 966) was negative with no BP-specific naive CD4⁺ T-cell lines detected. This donor did show only one positive T-cell line when using total CD4⁺ T cells. This discrepancy is probably due to the variability existing between the two experiments with this low number of cell lines.

Frequency of BP-specific naive CD4⁺ T lymphocytes was then calculated for each donor tested using the Poisson distribution law (Fig. 4A). The calculated frequency varied from 0 to 2.23 BP-specific naive T cells per million of naive CD4⁺ T cells corresponding to a mean value of 0.69.

To confirm that BP-specific T cells were from the naive compartment, memory CD4⁺ T lymphocytes characterized by the expression of CD45RO were also isolated from PBMCs of two healthy donors from the ten studied using

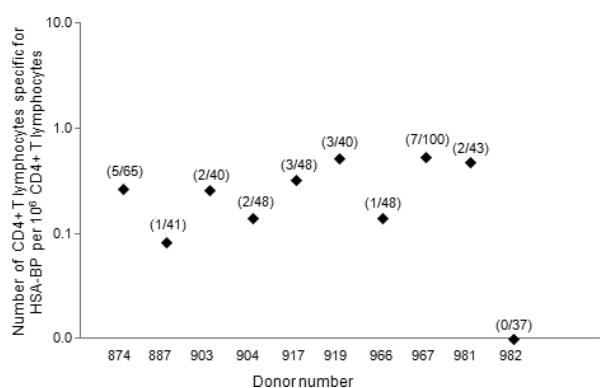


Figure 3 Frequency of human CD4⁺ T lymphocytes specific to HSA-BP. Frequency was calculated for each donor using the Poisson distribution law. On the x-axis is represented the donor's number. For the ten donors tested, the number of T-cell lines found specific to HSA-BP out of the number of T-cell lines tested is indicated in brackets.

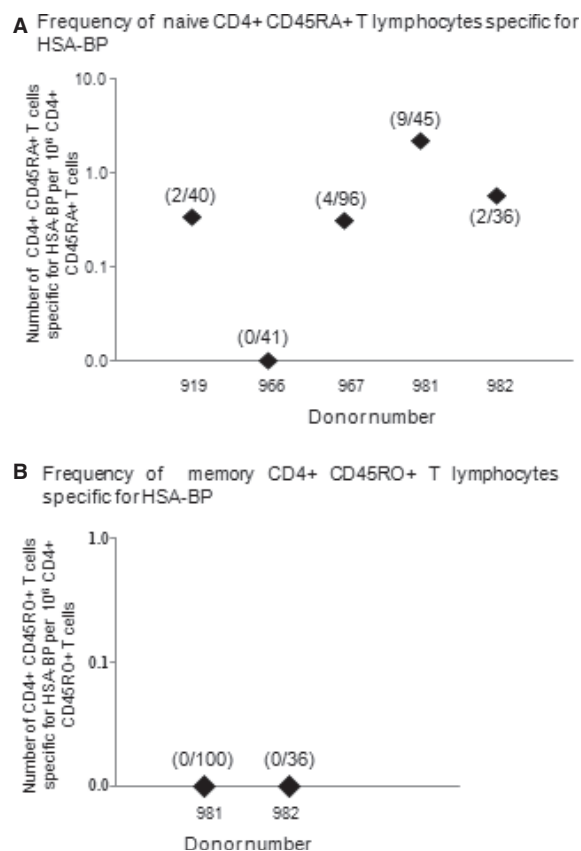


Figure 4 Naive CD4⁺ CD45RA⁺ T-cell lines specific to HSA-BP. (A) Frequency of CD4⁺ CD45RA⁺ T cells specific to BP. (B) Frequency of memory CD4⁺ CD45RO⁺ T cells. The frequency of T cells was calculated for each donor using the Poisson distribution law. On the x-axis is represented the donor's number. The number of HSA-BP-specific T-cell lines out of the number of T-cell lines tested is indicated in brackets.

the same methodology. For the two studied donors (donors 981 and 982), no memory BP-specific CD4⁺ CD45RO⁺ T-cell lines were detected (Fig. 4B), whereas BP-specific CD4⁺ T cells or BP-specific CD4⁺ CD45RA⁺ T cells were found.

Discussion

The objective of this study was to identify and analyze the presence of CD4⁺ T cells specific to BP in human healthy donors. CD4⁺ T cells were harvested from healthy donors to evaluate the capacity of BP bound by serum proteins to prime naive CD4⁺ T cells and to reproduce the *in vivo* induction of an immune response by BP-haptenized proteins. Measurement of anti-BP-specific IgE for each donor was performed to confirm their nonrecent sensitized status to exclude sensitized donors with possible allergy to penicillin (15–18). HLA class II polymorphism was also taken into account, and the ten donors tested harbored different HLA-DRB1 allotypes representative of the most frequent HLA-DRB1 allotypes present in the Caucasian population. T-cell stimulation was conducted in conditions, whereas DCs were more prone to induce a Th1 response.

In vitro amplification of BP-specific CD4⁺ T lymphocytes was performed using autologous DCs previously loaded with HSA-BP bioconjugates. T-cell frequency determination does not require identifying T-cell epitopes and HLA class II molecules, in contrast to techniques using HLA class II multimers (19, 20). Indeed, using antigen-presenting cell (APC) such as DCs (12, 13) or monocytes (21), the antigen tested is digested into peptides, presented through their HLA-II molecules to CD4⁺ T cells. This methodology has been previously used to determine the frequency of CD4⁺ T cells specific to different therapeutic antibodies (12) and recombinant erythropoietin (13).

In our study, we used HSA as the BP carrier protein. Human serum albumin is the most abundant protein in the serum, and past studies have shown the presence of HSA-BP in the serum of BP-allergic patients (7–10). Moreover, HSA-BP have been found to activate BP-specific T cells from allergic patients (6). Our HSA-BP bioconjugate synthesized at basic pH displayed at most nineteen BP molecules covalently bound per one HSA molecule, corresponding to a modification rate observed *in vivo*. Meng et al. (7) found fourteen BP-binding sites on HSA purified from a pool of plasma samples taken from BP-treated patients. For other β -lactam antibiotics such as flucloxacillin or piperacillin, drug–HSA bioconjugates have also been found in the serum of treated patients (22, 23).

Our results showed for the first time the presence of naive BP-specific CD4⁺ T cells in healthy donors. The mean frequency of BP-specific CD4⁺ T cells was 0.29 per million of CD4⁺ T cells. However, it is important to precise that this frequency can be underestimated because BP can also bind to other proteins, and free BP can also bind directly to HLA molecules. This frequency was in the same range as those found for well-known immunogenic therapeutic antibodies that induce the production of antidrug antibody in clinic,

after their administration to patients (24–28). Indeed, the frequencies were 0.35; 0.33, and 0.20 of specific CD4⁺ T cells per million of CD4⁺ T cells for rituximab, infliximab, and adalimumab, respectively (12). These results concerning preexisting T cells specific to therapeutic antibodies also showed a correlation between the size of the preexisting naive CD4⁺ T-cell repertoire specific to therapeutic antibodies and their potential immunogenicity (12).

When experiments were performed on naive CD4⁺ CD45RA⁺ T cells, BP-specific naive CD4⁺ CD45RA⁺ T cells were detected at a mean frequency of 0.69 BP-specific T-cells per million of naive CD4⁺ T cells, whereas no BP-specific memory CD4⁺ CD45RO⁺ T-cells were detected in healthy donors. The low frequency found for BP-specific T-cells reinforced our hypothesis that these T cells belong to the naive compartment. Indeed, previous works performed on patients allergic to amoxicillin showed a frequency varying from 30 to 125 drug-specific T lymphocytes per million of PBMCs (29) nearly 100- to 500-fold higher than our findings. This high frequency found for drug-memory T cells was a consequence of an important amplification of preexisting naive T cells specific to amoxicillin.

Benzylpenicillin-specific CD4⁺ or CD4⁺ CD4RA⁺ T cells were detected in all the healthy donors tested strongly, suggesting that most people can be sensitized to penicillin, but only a few individuals can mount an allergic response due to additional factors, providing necessary co-stimulation signals for T-cell activation. These additional factors are still poorly described, but a disruption of tolerance to foreign antigen, as described for nickel and other molecules, are a likely hypothesis (30).

Our study shows for the first time the existence of naive BP-specific T-cells in healthy donors. We were also able to quantify the frequency of these preexisting naive CD4⁺ T lymphocytes specific to BP using an *in vitro* T-cell assay. This study also showed the capacity of HSA-BP to be recognized by multiple donors, irrespective of their HLA typing. However, the possible correlation of the size of the naive T-cell compartment with the potential immunogenicity of drugs remains to be determined.

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Author contributions

CN designed the study, performed experiments and data acquisition, participated in data analysis and interpretation, and drafted the manuscript. SD took part in study conception, performed experiments and data acquisition, and participated in data analysis and interpretation. FH performed mass spectrometry experiments, data interpretation, and

manuscript drafting. LC performed measurement of anti-benzylpenicillin antibodies and data interpretation. RW and NC took part in study conception and manuscript drafting. DJ took part in study conception and participated in data interpretation and manuscript drafting. BM and MP designed the study, supervised the conduct of the study,

participated in data analysis and interpretation, and drafted the manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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