

Tracking Human Contact Allergens: From Mass Spectrometric Identification of Peptide-Bound Reactive Small Chemicals to Chemical-Specific Naive Human T-Cell Priming

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Modification of proteins by reactive small chemicals is a key step in the activation of chemical-specific T cells in allergic contact dermatitis (ACD). However, an integrated approach to characterize both the precise nature of chemically modified proteins and the chemical-specific T cells is currently lacking. Here, we analyze the molecular conditions for adduct formation of the strong human contact sensitizer 2,4-dinitrochlorobenzene (DNCB) and its water-soluble form, 2,4-dinitrobenzenesulfonic acid (DNBS), with both an all amino acid-containing model peptide ($\pm$ Cys) and the protein human serum albumin (HSA). Mass spectrometric detection and quantification revealed thiol-dependent peptide adduct formation at all pH values found in human skin layers. Highest modification rates were obtained with DNBS. Accordingly, DNBS- but not DNCB-modified human immature dendritic cells (iDC) induced *in vitro* primary human T-cell responses as did 2,4,6-trinitrobenzenesulfonic acid-modified iDC as measured by dinitrophenyl (DNP)- and trinitrophenyl (TNP)-specific T-cell proliferation and interferon gamma (IFN- γ) production in CD4⁺ and CD8⁺ T-cell subsets. Moreover, DNP-modified HSA protein effectively induced primary T-cell responses when processed by iDC. Thus, an integrated approach that combines efficient skin-related *in chemico* coupling analyses with an *in vitro* T-cell priming assay can be used to predict *in vivo* reactions of chemical contact allergens with extracellular and cellular proteins. This strategy supports the development of chemical-specific *in vitro* assays that are urgently required in predictive hazard identification and risk assessment of allergenic and nonallergenic chemicals.

Key Words: contact allergen; skin sensitization; T cell; mass spectrometry; haptenation; alternatives.

INTRODUCTION

Allergic contact dermatitis (ACD), a T cell-mediated inflammatory skin disease, results from the interaction of reactive small chemicals, so-called haptens, with proteins in the skin, concomitant innate inflammatory responses, and the subsequent chemical-specific recognition by the adaptive immune system (Freudenberg *et al.*, 2009; Martin *et al.*, 2006; Thierse *et al.*, 2005). T cells are the essential effector cells that trigger small chemical-specific immune responses in ACD (Askenase *et al.*, 2004; Martin *et al.*, 2006; Rustemeyer *et al.*, 2001; Sallusto, 2001; Sallusto *et al.*, 2000). Effector T cells in ACD are IFN- γ -producing CD4⁺ Th1 and CD8⁺ cytotoxic Tc1 cells as well as Th17 and Tc17 cells. Moreover, interleukin-10- and transforming growth factor- β -secreting CD4⁺ regulatory T cells (Treg) limit the extent and eventually down regulate the allergic response (Cavani, 2008; Martin, 2004; Vocanson *et al.*, 2008).

Although many details of contact allergen immune recognition and of the complex pathophysiology of ACD have been clarified, several aspects are still unknown or require further investigation. For example, chemical-specific naive T-cell frequencies and the interplay between effector T-cell and Treg responses are all important issues. Very little is known about the mechanisms of immunogenic chemical-peptide/-protein modification, which is regarded as a crucial step in sensitizing human T cells (Basketter *et al.*, 1997; Karlberg *et al.*, 2008; Lepoittevin, 2006). Reactive small chemicals must bind to proteins to form immunogenic T-cell epitopes. They can either modify extracellular and cellular proteins that must then be processed to chemical-modified major histocompatibility complex (MHC)-binding peptides or they can directly modify

The authors certify that all research involving human subjects was done under full compliance with all government policies and the Helsinki Declaration.

MHC-bound peptides on the cell surface (Martin, 2004; Martin *et al.*, 1992, 1993; Ortmann *et al.*, 1992; Padovan *et al.*, 1996, 1997; Weltzien and Padovan, 1998). In fact, both mechanisms seem to be of physiological relevance. Thus, chemical-specific T cells can be induced in a processing-dependent or -independent manner by human antigen presenting cells (APCs) (Brander *et al.*, 1995; Moulon *et al.*, 1995; Padovan *et al.*, 1996; Thierse *et al.*, 2004). Hence, T-cell immune reactions elicited by protein-bound small chemicals are complex but are always based on efficient chemical-protein interactions with two principal physiological target protein species, extracellular skin proteins or cell-associated proteins.

One of these potential target proteins is the human serum albumin (HSA). HSA is a multifunctional protein that is present in high concentrations in human skin and sweat. It serves as a carrier protein for a great variety of drugs and chemicals, including contact allergens. After contact allergen binding, it can be transformed into a functional mediator of chemical-specific human T-cell activation. Examples are penicillin G (Brander *et al.*, 1995), nickel (Thierse *et al.*, 2004, 2005), the most common human contact allergen, and *p*-phenylenediamine (PPD) (Jenkinson *et al.*, 2010), one of the most often encountered contact allergens present in numerous consumer products. In all three cases, chemical-specific T-cell responses were observed, suggesting a potent immunological role for HSA as a carrier protein that mediates chemical-specific T-cell responses in ACD. In order to better understand the chemical reactions of contact allergens with proteins or peptides, several bioanalytical approaches have been performed. For example, the chemical reaction of the strong contact sensitizer 2,4-dinitrochlorobenzene (DNCB) with HSA or the reactions of synthetic peptides with reactive small chemicals have been investigated. However, none of those studies integrated the chemical results and chemical-specific T-cell responses that underlie ACD (Aleksic *et al.*, 2007, 2009; Eilstein *et al.*, 2008; Gerberick *et al.*, 2007). Thus, the validated qualitative and quantitative analysis of DNCB-specific protein modification related to functional T-cell activation remains to be established.

In this study, we describe a comparable method of recently developed mass spectrometric approaches (Aleksic *et al.*, 2007, 2008) for the study of the *in vitro* modification of proteins or peptides with DNCB and 2,4-dinitrobenzenesulfonic acid (DNBS). After validation of thiol-specific DNCB and DNBS modification of a synthetic peptide carrying all amino acids, peptide-21, the method was applied to HSA and to cellular surfaces of human immature dendritic cells (iDC), both subsequently being implemented into a refined *in vitro* T-cell priming assay (TCPA). This assay allows the detection of chemical-specific T cells in the naive human peripheral T-cell population by measurement of proliferation and, at the single-cell level, of IFN- γ production in CD4⁺ and CD8⁺ T cells. Both chemical-modified iDC as well as contact allergens coupled to HSA were used to prime naive human T cells. The use of

chemical-coupled self-proteins allows a standardization of the test compounds and eliminates toxicity associated with solvents and/or test compounds when added directly to T-cell assays.

This integrated approach (Fig. 1) can now be used for the development of chemical-specific T cell-based assays including the *in vitro* TCPA proposed here. The TCPA may be useful for medical diagnostic or therapeutic purposes and, importantly, for the predictive *in vitro* identification of contact allergens and drugs that cause ACD and T cell-mediated adverse drug reactions. Such predictive assays are needed to eventually replace animal testing of chemicals in hazard identification and risk assessment strategies.

MATERIALS AND METHODS

Materials. Peptide-21 (LHKSMGRTWQFDYNPEACVIK) and peptide-20 (LHKSMGRTWQFDYNPEAVIK) were synthesized according to the method of

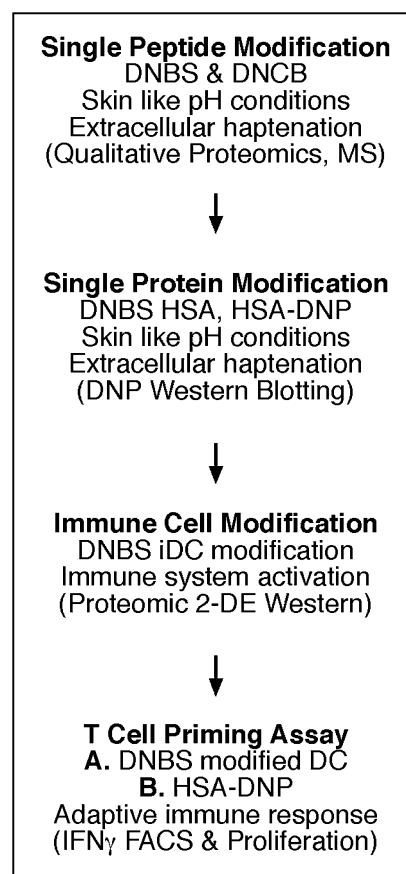


FIG. 1. From quality-controlled reactive small chemical-peptide interactions and chemical-modified proteins toward the development of novel *in vitro* TCPA. Integrated strategy for the assessment of small chemical reactivity with peptide and proteins *in vitro* and in cells and of the priming of naive T cells to these chemicals. The approach is applicable to medium throughput level, e.g., with complete immunoproteomic analyses of two to four or more chemicals in approximately 4 weeks, depending on resources being available.

Dormeyer *et al.* (2005). DNCB-, DNBS-, 2,4,6-trinitrobenzenesulfonic acid (TNBS)-, salicylic acid (SA)-, and DNP-specific primary polyclonal rabbit antibody were from Sigma-Aldrich. Secondary goat anti-rabbit HRP-conjugated antibody was from Cell Signaling. Polyvinylidene fluoride (PVDF) membrane was from Schleicher and Schuell. Coomassie Plus Protein Assay Reagent and SuperSignal West Pico chemiluminescent substrate were from Pierce. Dulbecco's modified eagle medium and fetal calf serum were obtained from Biocrom. IPG buffer, IPGphor, and electrophoresis chamber (Ettan DALT II system) were from GE Healthcare. FLA-5100 scanner was from Fujifilm-Lifescience. Spotpicker Proteineer spII, 800-384 AnchorChip target plate, and Ultraflex I MALDI-TOF/TOF were from Bruker Daltonics.

Peptide modification with DNBS or DNCB. DNCB, DNBS, and SA were diluted in 50mM ammonium acetate buffer to final working solutions of 10 and 100mM. All working solutions were freshly prepared and diluted to 1, 2.5, or 5mM final concentration and added to 0.05 mM final concentration of peptide-21 or of peptide-20. Solutions were prepared at pH values of 5.5, 6.8, and 7.4, with final chemical:peptide molecular ratios of 20:1, 50:1, or 100:1. Ratios were adapted and modified from chemical:protein ratios applied in the analytical study of Aleksic *et al.* (2007). One identical and additional lower ratios were chosen because under *in vivo* conditions in human skin—because of chemical-specific differential skin penetration and differential chemical distribution—higher and lower chemical:peptide or chemical:protein ratios have to be expected. Negative controls were performed with peptide-21 or peptide-20 using the same buffer conditions. Each incubation mixture was gently shaken for 24 h at 37°C. As equivalent results were obtained after 96 h incubations (data not shown), the reaction time was set to 24 h. The reaction was stopped by freezing the samples at −20°C. Incubations were performed in quadruplicates.

HSA modification with DNBS. DNBS (100 nmol, 5mM final concentration) was added to 20 µg HSA solution in 43.75mM ammonium acetate buffer at pH 5.5, pH 6.8, and pH 7.4 leading to a final ratio of 5:1 (nanomoles of chemical:micrograms of protein). In our pilot studies, this ratio was found to be optimal for protein modification after testing several ratios from 2.5 to 50 nmol DNBS/µg protein (data not shown). Samples were vortexed and incubated for 24 h at 37°C. The reaction was stopped by freezing the samples at −20°C. Incubation was performed in quadruplicates. Samples were used for standard one-dimensional gel electrophoresis SDS-PAGE protein separation (5 µg per lane).

Visualization of DNBS-modified proteins of viable iDC. Fifty micrograms of untreated iDC lysate and 50 µg lysate of viable iDC previously treated with 4mM DNBS for 10 min at 37°C were applied to 7-cm isoelectric focusing (IEF) gel strips (pH 3-10 non linear) for in-gel rehydration overnight at room temperature and focused according to the manufacturer's instructions (GE Healthcare). After IEF, the strips were prepared for the second dimension and transferred to 12% 7 × 8-cm nongradient gels. Electrophoresis was performed according to the biochemical standard protocols. Gels were either electroblotted or silver stained (Blum *et al.*, 1987).

Mass spectrometric detection of DNP-peptides. Prior to analysis, samples were diluted 1:20 in 0.1% trifluoroacetic acid (TFA), reducing the dimethyl sulfoxide content to a maximum of 0.05%. Samples were desalted and concentrated using a 10-µl C18 ZIPTIP pipette tips according to the manufacturer's instructions (Millipore). Samples were eluted with 1 µl α-cyano-4-hydroxycinnamic acid (HCCA) solution (saturated HCCA was diluted 1:7 [vol/vol] in 1:1 acetonitrile:0.1% TFA [vol/vol]) and spotted on a MALDI 800-384 AnchorChip target plate according to the manufacturer's instructions (Bruker Daltonics). Analysis was performed on an Ultraflex I MALDI-TOF instrument (Bruker Daltonics) using Bruker Flex-control, Flex-analysis, and Biotoools software. Calibration was performed externally using peptide calibration standard II (Sigma-Aldrich). MALDI mass spectra were recorded within an *m/z* range of 700–3500 in the positive ion reflector mode. Mass spectra were obtained by averaging up to 300 individual laser shots. Each analysis was performed in triplicate.

Immune detection of DNP-proteins. Gels were electroblotted on PVDF membranes using Biotec Fischer blotting apparatus. Blotting efficiency was controlled with Coomassie Brilliant Blue G250 staining. DNP modifications were detected by incubation with a DNP-specific primary polyclonal rabbit antibody at a dilution of 1:5000 followed by an HRP-conjugated goat anti-rabbit antibody, used at 1:2000. Chemiluminescence was detected using SuperSignal West Pico chemiluminescent substrate from Pierce.

Media. All cell culture reagents were from Invitrogen unless indicated otherwise. Cells were cultivated in RP5 (RPMI 1640) supplemented with 5% human AB serum (Cellgro, Mediatech, Inc.), 2mM L-glutamine with or without 100 U/ml penicillin and 100 µg/ml streptomycin, 25mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid buffer solution, and 10µM 2-mercaptoethanol. Cells were maintained at 37°C in a humidified incubator at 5% CO₂.

Cell isolation. Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood of healthy donors after informed consent. The protocols for this study were approved by the Ethics Committee of the University Medical Center, Freiburg, Germany. Alternatively, buffy coats were used. Ficoll-Hypaque (GE Healthcare) density gradient separation was performed. CD14⁺ monocytes were isolated by direct labeling with CD14 MicroBeads (Miltenyi Biotec GmbH). The CD14-negative fraction was frozen after isolation and thawed 1 day before addition of naive T cells. Naive T cells were isolated by a two-step protocol. First, T cells were isolated by depletion of non-T cells using the Pan T Cell Isolation Kit II (Miltenyi Biotec GmbH). Autologous CD3-depleted PBMC were treated with 25 µg/ml mitomycin C (Sigma-Aldrich) to avoid proliferation and were used as feeder cells in the cultures for the cytokine measurement. Second, the flow through fraction, containing T cells, was further depleted of CD25-expressing Treg and recently activated T cells as well as CD45RO-expressing memory T cells using CD25 MicroBeads and CD45RO MicroBeads according to the manufacturer's instructions (Miltenyi Biotec GmbH). The phenotype of naive T cells was determined by flow cytometry staining, with antibodies to CD3 (clone BW264/56), CD4 (clone M-T466), CD8 (clone BW135/80), CD45RA (clone T6D11) (Miltenyi Biotec GmbH), and CCR7 (clone 3D12, BD Bioscience). Purity of CD3⁺CD45RA⁺CCR7⁺ T cells exceeded 97%. For proliferation assays, T cells were cultivated for 1 day in RP5 before addition to iDC in the coculture.

Generation of iDCs. Isolated monocytes were differentiated for 7 days in Cellgro (Cellgenix) or X-Vivo15 (Lonza) supplemented with recombinant human granulocyte-macrophage colony-stimulating factor (500–1000 U/ml) and IL-4 (50–400 U/ml) (Provitro and Miltenyi Biotec GmbH) to iDC. iDC were either used fresh or frozen at day 7 or were frozen and stored at −70°C until used for restimulation of primary T-cell cultures. Surface markers on iDC (CD14/80/86/83 low and CD1a high) (CD14 clone M5E2, CD80 clone L307.4, CD86 clone 2331, CD83 clone HB15e, and CD1a clone HI149; all BD Biosciences) were analyzed using a FACScan instrument and CellQuest Pro software (BD Biosciences).

iDC modification with contact allergen. DNBS was diluted in sterile PBS, pH 7.4, to a stock solution of 4mM, as adapted from many studies from the early days of hapten-mediated immune reactions and was also found to be optimal by us. Therefore, this concentration was used in our study. TNBS was diluted with PBS to attain a 3mM solution. For direct modification, iDC were incubated either for 10 min with 4mM DNBS at 37°C or for 7 min with 3mM TNBS at 37°C. After extensive washing, iDC were plated in 96-well plates (2 × 10⁴ cells per well) or used for restimulation of primed T cells. DNCB was always freshly dissolved in acetone to a 100mM stock solution. iDC were modified for 10 min at 37°C with 5mM DNCB diluted in PBS.

T-cell proliferation assay. Assays were set up in round-bottom 96-well plates (Greiner Bio-One) with 2 × 10⁴ iDC per well either directly modified or preincubated for 24 h with DNCB dissolved in Cellgro at nontoxic concentrations or with solvent control. The solvent acetone was kept constant at 0.02%. To achieve complete iDC activation in the case of haptenated protein

addition, 10 ng/ml lipopolysaccharide (*Salmonella minnesota* R595; Calbiochem) was added after 24 h. Naive T cells (1×10^5) were added 24 h after iDC plating and cocultivated for 5–7 days. At days 5–7, assays were labeled for 18–20 h with 1 μ Ci [^{3} H]-thymidine (PerkinElmer) and harvested onto GF/C Filter plates using Packard FilterMate (PerkinElmer). Plates were dried, and scintillation fluid (MicroScint; PerkinElmer) was added. Counts were measured in a β -counter (TopCount; PerkinElmer).

To analyze the chemical specificity of the T-cell response, 2×10^4 viable freshly prepared DNBS- or TNBS-modified iDC were added at day 10 to the T-cell assay, and the chemical-specific secondary response was measured as T-cell proliferation 3 days later.

Detection of IFN- γ -producing *in vitro* primed T cells. Naive T cells were mixed with autologous feeder cells and chemical-modified iDC at a ratio of 10:5:1. The cells were cultured at 1×10^6 naive T cells/ml in RPMI 1640 + 5% AB serum for 10 days in 96-well plates with 100 μ l per well. On days 2, 4, and 6, recombinant IL-2 (R&D Systems), IL-7 (Miltenyi Biotec GmbH), and IL-15 (Miltenyi Biotec GmbH) and fresh cell culture medium were added. On day 9, the *in vitro* primed T cells were harvested, pooled, washed, and resuspended in culture medium + 5% AB serum and further cultured without addition of cytokines. After a 24-h cytokine-free cultivation of the T cells, thawed and freshly chemical-modified or unmodified iDC were added at a ratio of 10:1 for 6 h. For the last 4 h, 1 μ g/ml Brefeldin A (Sigma-Aldrich) was added to prevent cytokine secretion. The cells were harvested and fixed with Inside Fix (Miltenyi Biotec GmbH). The cells were stained intracellularly by Inside Perm (Miltenyi Biotec GmbH) and antibodies against IFN- γ (clone 45-15), CD4, CD8, and CD3 (Miltenyi Biotec GmbH). Flow cytometric analysis was performed using a MACSQuant and a FACSCalibur instrument and MACSQuantify (Miltenyi Biotec GmbH) and CellQuest (BD Biosciences) software, respectively.

Blocking of MHC/TCR interaction. TNBS-modified iDC were preincubated with 100 μ g/ml anti-MHC class I antibody (clone W6/32) and 100 μ g/ml anti-MHC class II human leukocyte antigen system-DR (HLA-DR) antibody (clone AC122; Miltenyi Biotec GmbH) 30 min prior to addition of *in vitro* primed T cells.

Statistics. Significance of HSA and peptide modification rates by contact allergens was calculated using one-way ANOVA combined with Bonferroni's Multiple Comparison Test (Prism 4.0). *p*-values of ≤ 0.05 were considered as statistically significant.

RESULTS

Chemical-peptide modification

Microenvironmental conditions trigger innate and T cell-mediated allergic immune responses in skin. To examine the potential influence of the pH values found in the different layers of human skin human on chemical modification of the cysteine (Cys)-containing model peptide-21 and Cys-free control peptide-20 by the strong contact sensitizers DNBS and DNCB, adduct formation was assessed based on calculated mass shifts by MALDI-TOF mass spectrometry. As demonstrated in Figure 2, treatment with DNBS (Fig. 2A) and DNCB (Fig. 2B) resulted in new mass spectrometric signals corresponding to the 166 *m/z* mass shift of peptide-21 at all skin relevant pH conditions tested (pH 5.5, pH 6.8, and pH 7.4). Semiquantitative information derived from MALDI-TOF spectra indicated that the percentage of adduct formation by DNBS with peptide-21 increased with increasing pH (Fig. 3A). DNCB-modified peptide-21 showed

the highest adduct formation rates at pH 6.8 (Fig. 3B). As the chemical to peptide ratio of 20:1 was most effective in comparison with modification results using ratios of 50:1 or 100:1 (data not shown), this ratio was applied for all peptide studies. Moreover, no peptide modification was observed with the Cys-free variant of peptide-21 (peptide-20) (Figs. 2C and 2D). The skin irritant SA was used as a noncontact allergen control, with no modification of peptide-21 or peptide-20 detected after incubation with SA (data not shown). In summary, our data clearly show pH-dependent differential DNBS- and DNCB-specific peptide adduct formation and selective modification of Cys in model peptide-21.

Chemical-protein modification

After demonstrating DNBS/DNCB-peptide interactions by mass spectrometry, chemical modification of an immunologically relevant skin protein as well as chemical modification of multiple cellular proteins were evaluated. As demonstrated by Western blotting, HSA was efficiently modified *in vitro* by DNBS (Fig. 3C). Semiquantitative analyses of HSA-DNBS adducts (HSA-DNP) show a significant increase in the modification rate (pH 5.5 vs. pH 7.4, *p*-value = 0.011) according to increasing pH values (Fig. 3D) as already observed for peptide-21.

Moreover, we adapted the *in vitro* protocols from peptides and proteins to viable cells that were modified with DNBS. Thus, we were able to detect DNP-modified cellular proteins in human monocyte-derived iDC. Only 15 DNP-specific protein modifications were detected by two-dimensional gel electrophoresis (2-DE) and 2-DE Western blotting (Fig. 3F). These data clearly indicate that of the hundreds of cellular proteins expressed in human dendritic cells (DCs), only a limited number is targeted by DNBS (Fig. 3E). The successful modification of cellular proteins is a prerequisite for the generation of T-cell epitopes.

Establishment of an *in vitro* TCPA to detect T-cell responses to chemicals

We next assessed whether the direct modification of iDC with contact allergens and modification of cellular proteins in fact result in the formation of T-cell epitopes. Therefore, we used hapten-modified iDC to test for the presence of contact allergen-specific T cells within the naive human T-cell repertoire. We established an *in vitro* TCPA using purified naive human T cells and DNBS- and TNBS-modified iDC and, in addition, chemical-modified self-proteins such as HSA as the antigen source (Fig. 4A). With this assay, we were able to identify chemical-specific T cells in the naive repertoire by measurement of proliferation or of IFN- γ production in the CD4 $^+$ as well as CD8 $^+$ T-cell subset at the single-cell level (Fig. 5).

Readout (1): proliferation

T-cell proliferation was induced by iDC modified with the contact allergens DNBS and TNBS (Fig. 4B) but not by unmodified or DNCB-modified iDC. Restimulation of the

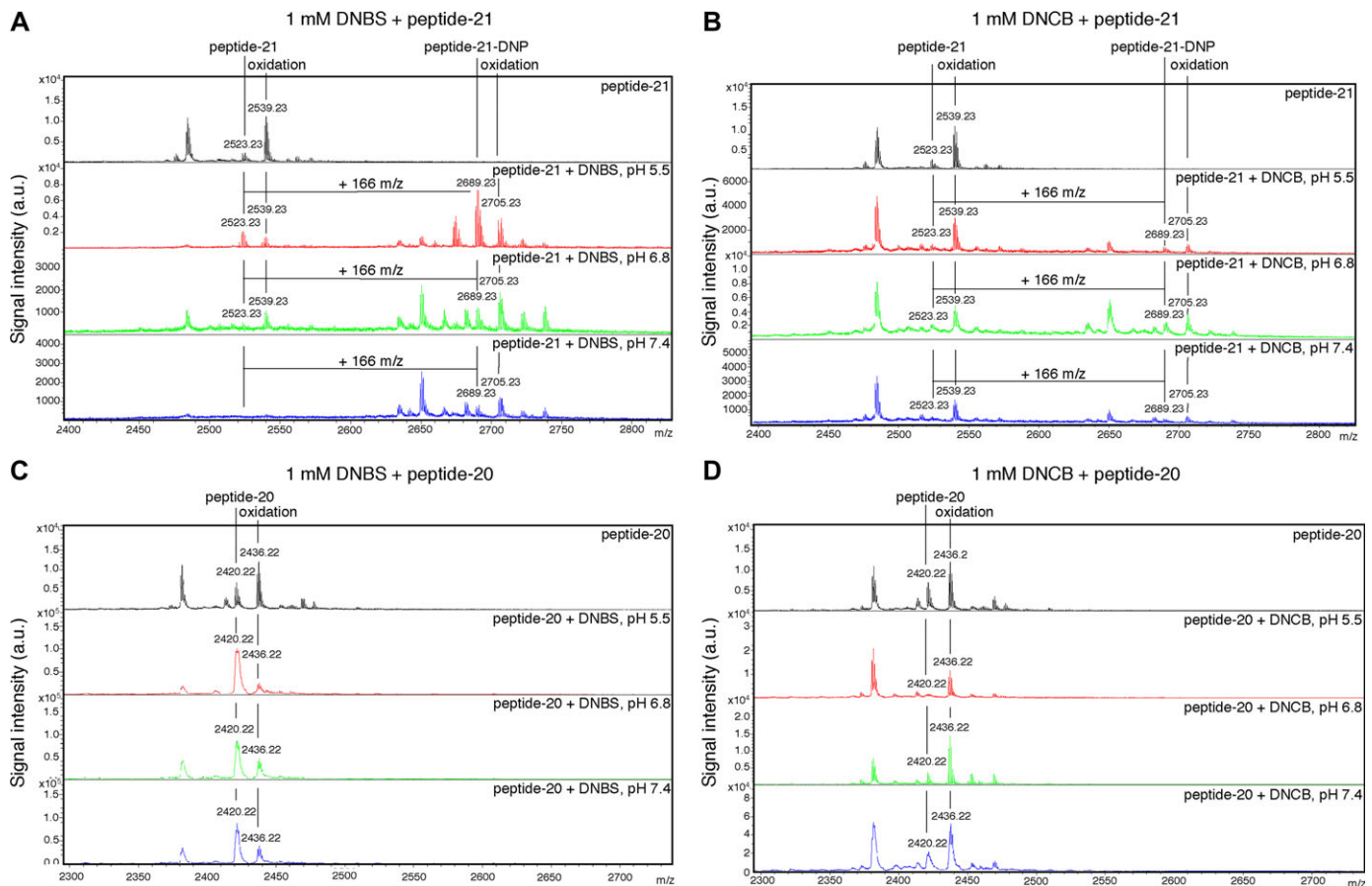


FIG. 2. Detection of chemical-peptide modification results by mass shift analysis. MALDI-TOF mass spectrometric analysis of (A) DNBS-induced and (B) DNCB-induced adduct formation with cysteine-containing model peptide-21 showed new DNP-specific peptide signals (peptide-21-DNP) at skin-related pH conditions: pH 5.5 (*stratum corneum*), pH 6.8 (*stratum basale*), and pH 7.4 (physiological). Mass spectrometric analysis of (C) DNBS-induced or (D) DNCB-induced adduct formation with cysteine-free control peptide-20 showed no modification signals regardless of pH condition. Data are representative of three independent experiments. Signal intensity is given in arbitrary units (au).

cultures with modified iDC revealed that the T-cell response is clearly hapten specific (Fig. 4C). Thus, T cells primed with TNBS-modified iDC responded strongly to TNBS-modified iDC but only weakly to DNBS-modified iDC upon secondary stimulation and vice versa. Because we have demonstrated efficient modification of HSA with DNBS, we used this haptenated protein as antigen in the TCPA, thus mimicking the uptake and processing of hapten-modified skin proteins by DC in ACD. Priming of T cells with iDC presenting processed HSA-DNP resulted in a significant dose-dependent T-cell response in contrast to iDC presenting unmodified HSA (Fig. 6A). In contrast, iDC preincubated with DNCB at nontoxic concentrations for 24 h failed to induce T-cell proliferation (Fig. 6B), indicating inefficient modification of cellular proteins by DNCB at pH 7.4 in the culture medium.

Readout (2): IFN- γ production

To enable analysis of the chemical-specific T-cell response on a single-cell basis, we evaluated CD4⁺ and CD8⁺ effector

T-cell responses upon priming of naive sorted T cells and secondary stimulation with autologous chemical-modified iDC by intracellular cytokine staining. Because we observed in a pilot study (data not shown) that *in vitro* primed T cells efficiently produced IFN- γ in response to contact allergen, we restricted our analysis to this cytokine.

Naive T cells of three donors were primed *in vitro* by cultivation with autologous DNBS-modified, TNBS-modified, or unmodified iDC for 10 days in the presence of IL-2, IL-7, and IL-15 to enhance expansion. To assess the presence of activated and expanded DNP- or trinitrophenyl (TNP)-specific CD4⁺ and CD8⁺ T cells in the cultures, the cells were restimulated with DNBS-modified, TNBS-modified, or unmodified iDC for 6 h and intracellular IFN- γ was analyzed.

When naive unprimed T cells were stimulated for 6 h with chemical-modified iDC, no enhanced frequency of IFN- γ ⁺ T cells was detected, indicating that no preactivated chemical-specific T cells were present in our separated naive T-cell population (Fig. 5A). For one of three donors, 2% of the CD4⁺

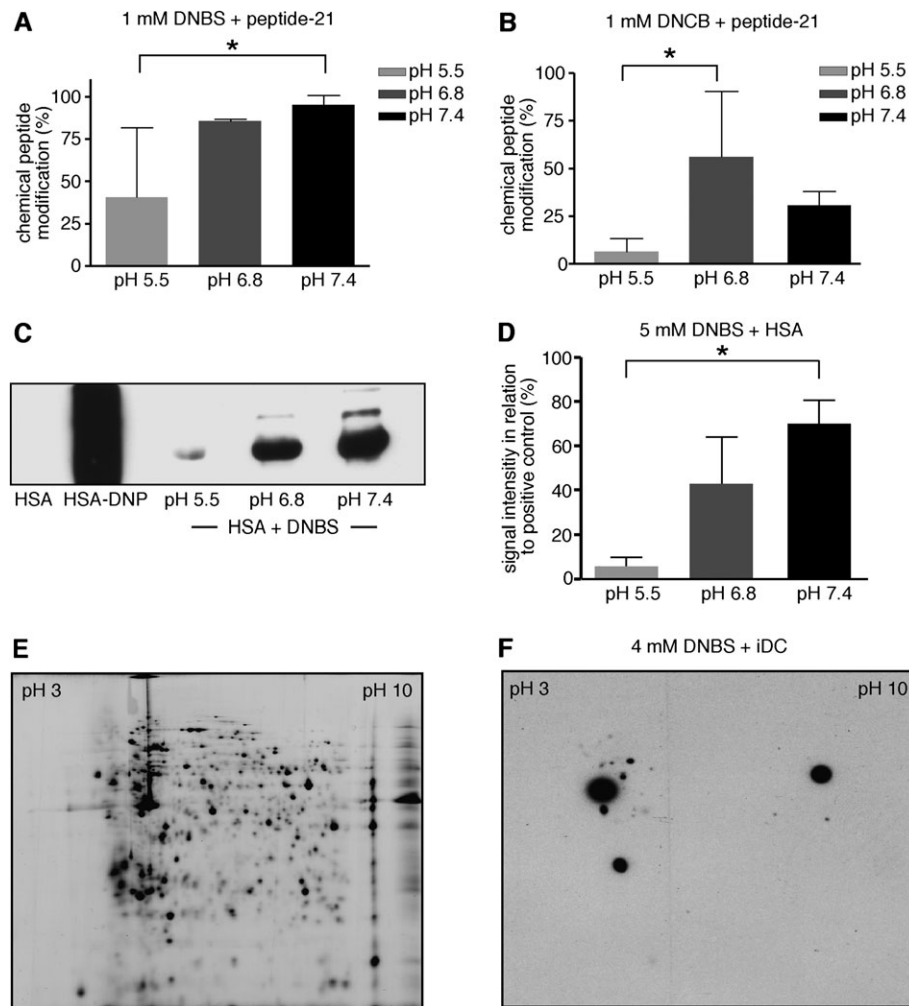


FIG. 3. Semiquantitative analysis of chemical-peptide modification and detection of chemical-protein modification in HSA and human DCs. (A) Peptide-21 adduct formation of DNBS ($*p = 0.02$) and (B) of DNCB ($*p = 0.02$) at skin-related pH conditions: pH 5.5 (*stratum corneum*), pH 6.8 (*stratum basale*), and pH 7.4 (physiological). Sum of χ^2 of new signals (covalent adducts) was set in relation to the total sum (χ^2) of all signals (100%). Statistical analysis was performed using one-way ANOVA combined with Bonferroni's Multiple Comparison Test. (C) HSA was modified with DNBS at skin-related pH conditions and analyzed by Western blotting with anti-DNP antibody. Unmodified HSA (first lane) and commercial HSA-DNP (second lane) were used as controls. (D) DNP-specific signal intensities were quantified by densitometry. (E) Human iDC were modified with DNBS at physiological pH 7.4 and lysed, and iDC proteins were separated by 2D gel electrophoresis (silver staining). (F) Western blotting with anti-DNP antibodies of (C) reveals DNP-modified proteins in human iDC. Data are representative of three independent experiments.

T cells cultivated with DNBS-modified iDC reacted with IFN- γ production upon reactivation with DNBS-modified iDC (Fig. 5A). For the three donors 1.7, 0.4, and 0.2% of CD8 $^{+}$ T cells, respectively, were detected after priming and restimulation with DNBS-modified iDC, whereas less than 0.1–0.3% of T cells produced IFN- γ in response to unmodified iDC (Figs. 5A and 5B). When primed and restimulated with TNBS-modified iDC, two donors showed specific CD4 $^{+}$ and all donors showed specific CD8 $^{+}$ T-cell responses. Among CD4 $^{+}$ T cells 3.9 and 9.5% and among CD8 $^{+}$ T cells 10.2, 3.4, and 0.4% IFN- γ^{+} T cells were found after specific restimulation. Less than 0.1% produced IFN- γ upon restimulation with unmodified iDC (Figs. 5A and 5B). To provide assurance about the antigen specificity of the primed T cells as well as the specificity of the

detection method, we performed restimulations of DNBS-primed T cells with TNBS-modified iDC and vice versa. Only 0.41% of CD4 $^{+}$ and 0.34% of CD8 $^{+}$ T cells primed with DNBS-modified iDC produced IFN- γ when restimulated with TNBS-modified iDC (Figs. 5A and 5B; donor 1). Among T cells primed with TNBS-modified iDC and restimulated with DNBS-modified iDC, 5% of CD4 $^{+}$ and 0.21% of CD8 $^{+}$ produced IFN- γ (Fig. 5B; donor 2). This may indicate that a minor fraction of the primed T cells is cross-reactive for DNP and TNP, but the vast majority of T cells showed specificity for either DNP or TNP. The restimulation of *in vitro* primed T cells was found to depend on MHC/T-cell receptor (TCR) interaction as blocking of MHC class I and HLA-DR reduced the frequency of IFN- γ -producing cells. In

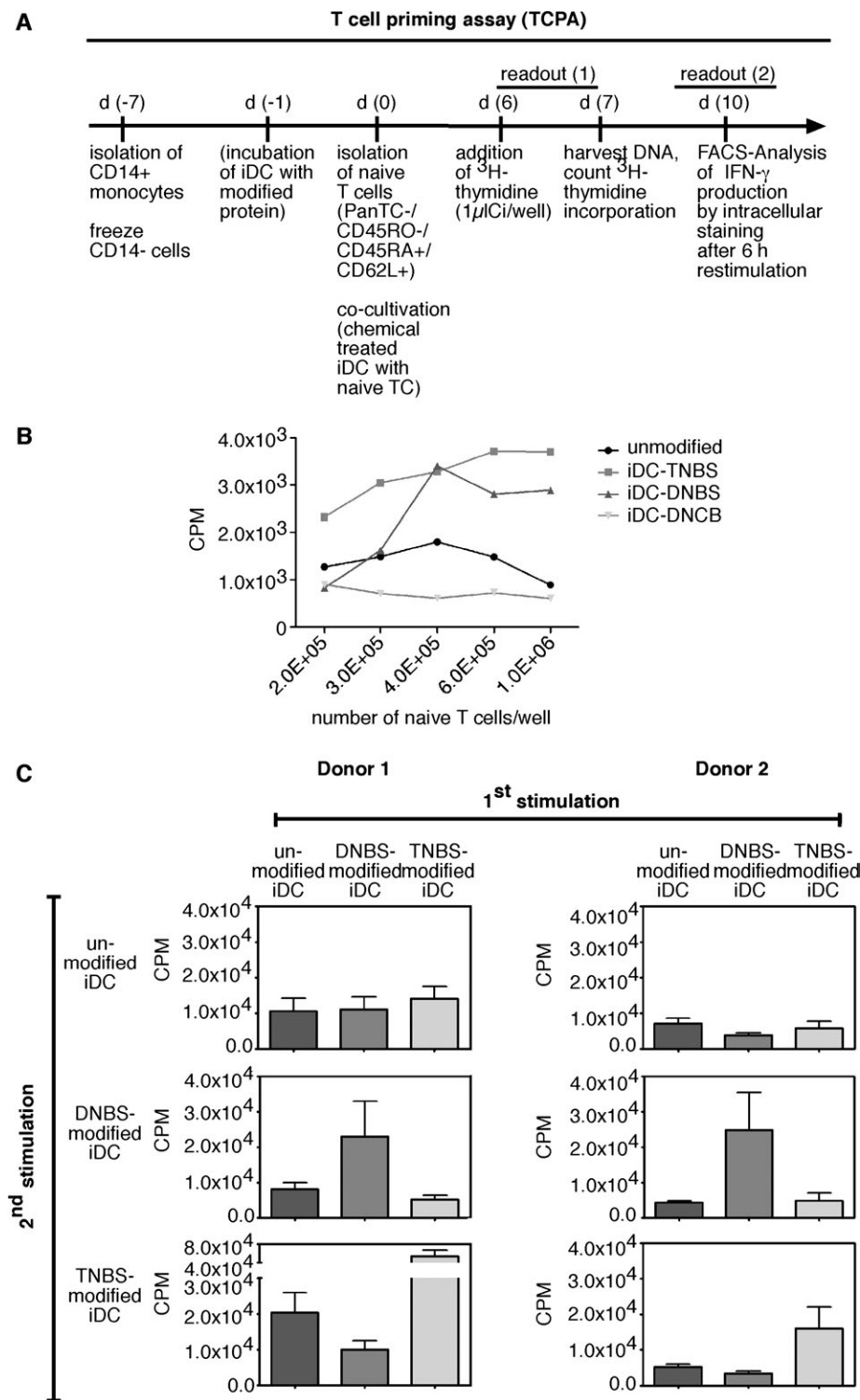


FIG. 4. Chemical-modified DC prime naive human T cells. (A) Protocol for the *in vitro* TCPA and time points of proliferation, readout (1), and IFN-γ secretion, readout (2). (B) Sorted naive human T cells were primed with chemical-modified (TNBS, DNBS, or DNCB) iDC (one representative donor of three is shown). Proliferation was measured on day 7 by ³H-thymidine incorporation. (C) The chemical specificity and donor dependence of the T-cell response were analyzed as shown for two different donors. Primary cultures were restimulated on day 10 with DNBS- or TNBS-modified iDC in a crisscross experiment. Data represent mean counts per minute (CPM) values of triplicate measurements ± SD.

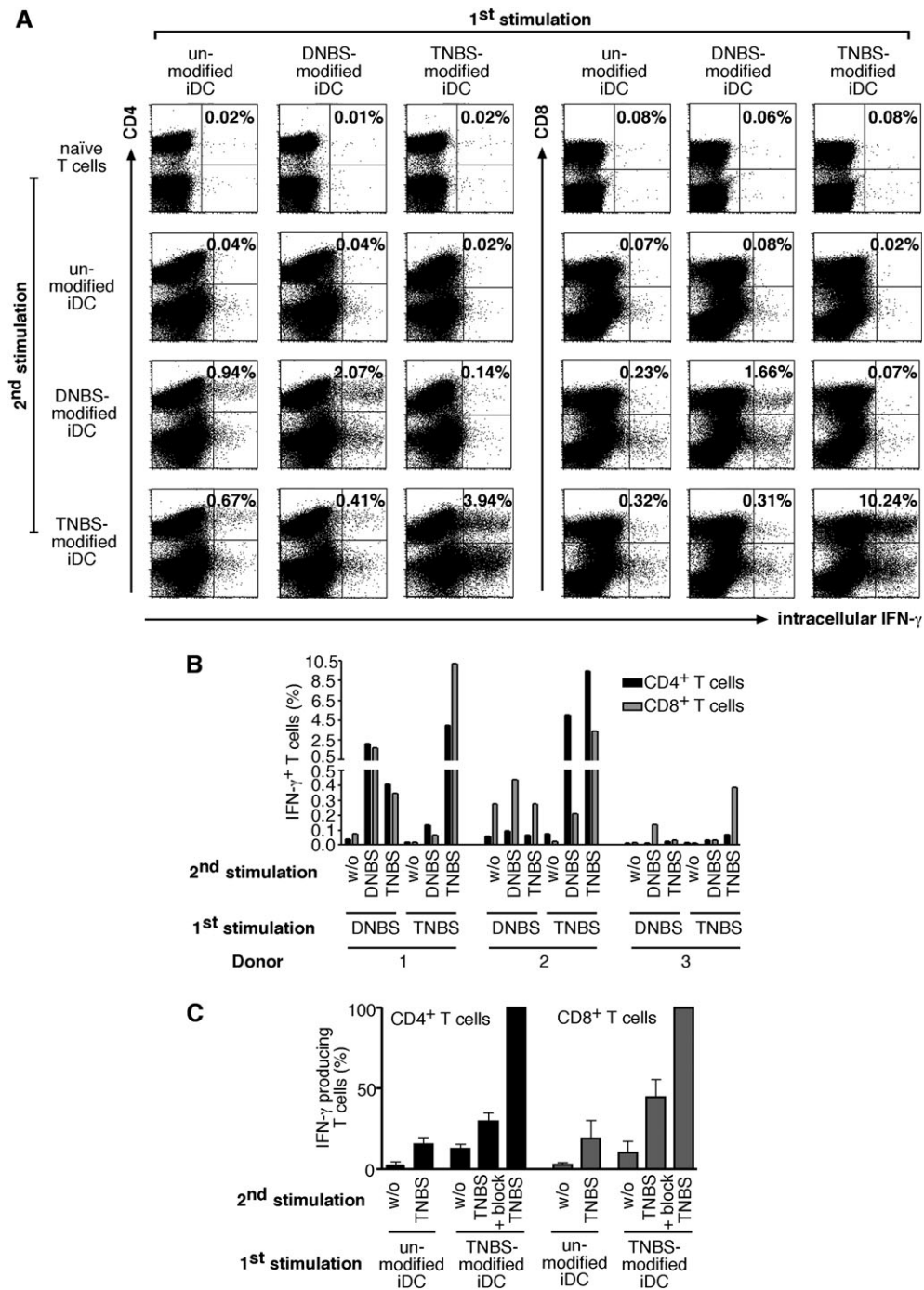


FIG. 5. Induction of hapten-specific IFN- γ -producing effector T cells with directly hapten-modified iDC. (A–C) Naïve T cells were cultured with DNBS- or TNBS-modified iDC for 10 days, restimulated with the same chemical-modified iDC or crisscross with either DNBS- or TNBS-modified iDC for 6 h, and subsequently analyzed for IFN- γ production by intracellular staining. Numbers indicate the frequencies of IFN- γ ⁺ cells among CD4⁺ or CD8⁺ T cells. (A) Flow cytometric analysis of intracellular IFN- γ of one representative donor of three. (B) Results of three donors. (C) Anti-MHC class I and anti-HLA-DR blocking antibodies were added during restimulation. Frequency of IFN- γ ⁺ T cells upon priming and restimulation with TNBS-modified iDC without antibodies was set as 100% ($n = 3$).

the presence of anti-MHC antibodies, only 30% of TNP-specific CD4⁺ T cells and 44% of TNP-specific CD8⁺ T cells expressed IFN- γ compared with the samples without blockade of MHC/TCR interaction (Fig. 5C).

In summary, these results confirm that the analysis of IFN- γ production at the single-cell level allows the identification of *in vitro* primed DNP- and TNP-specific CD4⁺ as well as CD8⁺ T cells.

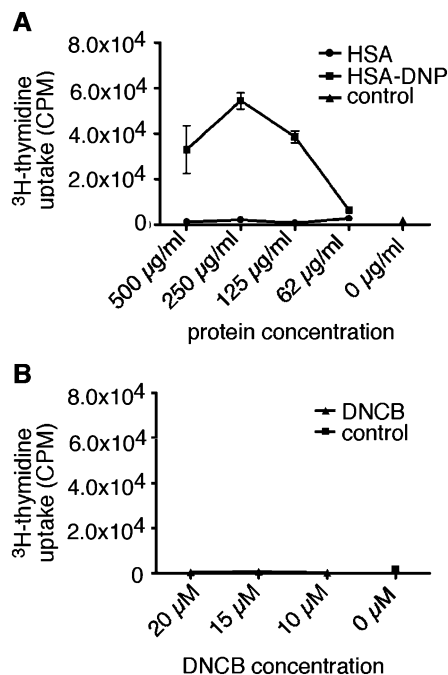


FIG. 6. Chemical-modified HSA conjugates (HSA-DNP) activate chemical-specific T cells in the naive T-cell repertoire. (A) iDC were incubated with HSA-DNP or unmodified HSA as control at the indicated concentrations, and sorted naive T cells were added. Proliferation was measured as in Figure 4B. (B) In addition, unmodified iDC (control) or iDC preincubated with the indicated DNCB concentrations for 24 h were used for T-cell priming. Sorted naive T cells were added after washing of the iDC, and T-cell proliferation was measured as described in Figure 4B. One representative donor of three is shown. Data represent mean CPM values of triplicate measurements $\pm$ SD.

DISCUSSION

Chemical modification of skin or cellular proteins and subsequent processing to antigenic hapten-modified MHC-binding peptides are required for T-cell epitope formation in ACD. Although our chemical and immunological understanding of molecular processes in skin sensitization has advanced significantly in recent years, most approaches have focused on assay-optimized, qualitative, or quantitative *in chemico* reactivity studies or distinct T-cell activation analyses without combining both (Aleksic *et al.*, 2007, 2008; Geiger *et al.*, 2009; Gerberick *et al.*, 2004, 2007; Guirionnet *et al.*, 2000; Krasteva *et al.*, 1996; Martin *et al.*, forthcoming; Natsch and Gfeller, 2008). In this study, we present a systematic two-step approach for evaluating the immunogenic potential of chemicals: first, validated analysis of skin-related chemical-peptide reactivity by mass spectrometric detection of adduct formation and second, chemical-specific naive human T-cell priming by iDC modified directly with contact allergens—under coupling conditions similar as studied with peptides—or fed with quality controlled HSA-contact allergen complexes.

Because it is known that hapten reactions of electrophilic small chemicals with nucleophilic amino acid side chains of

peptides or proteins are highly dependent on surrounding pH values, *in vitro* conditions are often set to optimize chemical side chain reactivities, e.g., pH 10.2 or pH 10.5 for Lys reactions based on its pK_a and to deprotonate the primary amine to make it available for covalent adduct formation (Gerberick *et al.*, 2004, 2007). However, single amino acid pK_a value may vary considerably, being significantly influenced by temperature, ionic strength, and particularly the microenvironment of ionized groups in three-dimensional molecules, in a specific compartment and/or state. One described example is Lys166 in HSA with a diminished pK_a value and thus a changed reactivity profile depending on the local physiological situation (Gerberick *et al.*, 2004). In the present study, we have investigated the impact of skin-related pH conditions for the modification of model peptide-21 and of model protein HSA with the contact allergens DNCB and DNBS. Treatment of the model peptide-21, composed of all amino acids, with DNCB and DNBS revealed exclusive thiol-dependent Cys modification for both contact allergens at all pH values tested, corresponding to the skin surface (pH 5.5), *stratum basale* in the epidermis (pH 6.8), and physiological pH (pH 7.4) (Ohman and Vahlquist, 1994). Usage of synthetic peptides containing all the proteinogenic amino acids $\pm$ cysteine gives an initial reactivity profile of chemicals examined in this study, thus allowing to instantly identify potential chemical-specific modified amino acid(s), e.g., cysteine, lysine, or tyrosine by mass spectrometry. Moreover, reactivity profiles may help to explain and classify chemical-specific T-cell responses or nonresponses. Highest relative semiquantitative DNCB peptide-21 modification rates were obtained at pH 6.8, although the efficiency of DNBS peptide-21 modifications increased as a function of increasing pH values of the incubation medium. Thus, as no solubility differences of peptides or chemicals were observed and equivalent buffer systems, temperature, and incubation times were applied, differential pH values seem to significantly create microenvironmental conditions, which favor a chemical-specific reaction or not. Because structure and function of biological molecules are known to be highly dependent on surrounding pH values, leading to more (at higher pH) or less deprotonated (at lower pH) reactive side groups (e.g., lowering the pH value from 7 to 5 leads to a 100-fold increase of H^+ and a 100-fold decrease of H^+ and vice versa), this partially will explain why electrophilic chemicals react much better at higher pH values with deprotonated molecules than under acidic conditions. Comparable pH-dependent mass spectrometric results were obtained by Aleksic *et al.* (2007) after HSA and DNCB incubation, also resulting in Cys-specific modification (Cys-34), but including additional allergen HSA modification sites of Lys, Tyr, His, and the N-terminus, whereas DNBS was not investigated in this study (Aleksic *et al.*, 2007). However, considering differential microenvironmental effects and other modification parameters such as prolonged chemical-peptide incubations, additional DNCB/DNBS-peptide adducts may occur after incubation with peptide-21, probably with more

rapidly modified Cys residues as compared with kinetic data of DNCB-modified Lys and Tyr residues (Aleksic *et al.*, 2009; Gerberick *et al.*, 2007). Yet, because in our study best chemical-peptide modification rates were obtained with DNBS in comparison with DNCB, we focused in the following preferentially more on DNBS-protein and DNBS-cell modifications than on DNCB-protein and DNCB-cell reactions.

Our surprising finding that DNBS modification of whole human APCs results in only a limited number of detectable DNP-modified cellular proteins may also be explained in part by the primary targeting of Cys thiol groups in peptide-21. Similar results were reported for human PBMC modified with radiolabeled DNCB (Pickard *et al.*, 2007), for human skin, and monocytic THP-1 cells (Megherbi *et al.*, 2009; Pickard *et al.*, 2009). The identification of such DNCB/DNBS-modified target proteins remains an important and challenging task. Chemical targeting of a rather limited number of proteins may be because of limited accessibility of some cellular proteins and of modifiable amino acids. As mentioned, the efficiency of protein modification—and consequently the sensitizing potential of the chemical—will be predominantly influenced by the specific target protein structures as well as by various cell compartments and other biochemical and physiological variables of the different skin layers. Such variables, including changing *in situ* pH values, will selectively impact on the chemical-specific reaction optimum (Basketter *et al.*, 1997; Martin *et al.*, 2006; Smith Pease, 2003). In particular, as seen with peptide-21, distinct DNCB-specific thiol modification with an optimum reactivity at pH 6.8 may limit the number of detectable cellular protein modifications. In addition, this may also affect the DC-dependent priming of T cells because it has been shown that allergen-induced thiol modifications trigger inflammatory cell responses (Bruchhausen *et al.*, 2003; Hirota *et al.*, 2009; Kagatani *et al.*, 2010; Suzuki *et al.*, 2009).

Chemical modification of carrier protein HSA with DNBS showed the highest rates of modification at pH 6.8 or pH 7.4, similar to our findings with peptide-21. These results match recent observations from human skin organ cultures. It was demonstrated that DNCB, after penetration of the *stratum corneum*, preferentially modifies the deeper skin layers, corresponding to pH values of 6.8 or higher (Pickard *et al.*, 2009). This suggests a chemical-specific *in vivo/in vitro* reactivity optimum, which may definitely vary from theoretical chemical predictions.

We have shown that both DNBS-modified iDC and chemically modified HSA can be used to prime human naive T cells as measured by IFN- γ production in CD4⁺ and CD8⁺ T-cell subsets at the single-cell level and by proliferation. Our results with HSA-DNP clearly indicate that contact allergen-modified self-proteins are useful reagents to detect chemical-specific T-cell responses in the naive human T-cell repertoire.

Stimulation with DNCB alone failed to induce such T-cell responses, which is inline with the inefficient peptide-21

modification at pH 7.4 by DNCB compared with DNBS. As a consequence, DNCB may not generate T-cell determinants efficiently in the culture medium at pH 7.4. Our data confirm previous human T-cell studies, showing no responses when naive T cells from nonsensitized donors were stimulated with DNCB (Pickard *et al.*, 2007).

We extend previous studies with allergic patients showing chemical-specific *in vitro* T-cell responses to HSA conjugated to penicillin G (Brander *et al.*, 1995) or the contact allergens nickel (Thierse *et al.*, 2004, 2005) and PPD (Jenkinson *et al.*, 2010). Remarkably, HSA-PPD induced a more potent proliferative signal than PPD alone (Jenkinson *et al.*, 2010). We now show for the first time that contact allergen-modified HSA conjugates can also be used to identify chemical-specific T cells in the naive T-cell repertoire. This underscores the importance of further qualitative and quantitative characterization of small chemical-protein interactions. It also supports the implementation of chemical-modified self-proteins such as HSA into novel chemical-specific predictive TCPAs for contact allergen identification.

A major advantage of such conjugates is the mass spectrometric quality control of the reagent that is used for T-cell priming. Direct modification of APC is difficult to standardize. Only in rare cases are there contact allergen-specific antibodies that permit the detection and quantification of the APC modification. This precludes the interpretation of negative results in T-cell assays. In addition, the use of chemical-HSA conjugates eliminates the problems associated with direct addition of chemicals to the *in vitro* assays because of frequently encountered toxicity of the test compound and/or the solvent, which often prevents T-cell priming.

The development of chemical-specific T-cell assays based on strategies outlined here can be used to characterize the type of responder T cell at the single-cell level based on cytokine production and surface marker phenotyping in multiparametric flow cytometry analyses. Frequencies and the TCR repertoire of chemical-specific T cells can be assessed. T-cell lines and clones can be established by isolation of chemical-specific T cells, e.g., by using the cytokine secretion technology. Amplification steps can be integrated to expand rare naive precursor T-cell populations for subsequent analysis of their functional phenotype and TCR repertoire studies as demonstrated for protein allergens (Geiger *et al.*, 2009). Most importantly, the development of refined *in vitro* TCPAs by using chemical-coupled self-proteins as standardized antigens will be useful for the predictive *in vitro* identification of contact allergens.

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