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# Detection of low avidity desmoglein 3-reactive T cells in pemphigus vulgaris using HLA-DRβ1*0402 tetramers

**Christian Veldman [a,*], Rüdiger Eming [a], Sonja Wolff-Franke [a], Grete Sonderstrup [b], William W. Kwok [c], Michael Hertl [a]**

[a] *Department of Dermatology and Allergology, University of Marburg, Deutschhausstrasse 9, D-35033 Marburg, Germany*
[b] *Department of Microbiology and Immunology, Medical School, Stanford University, Stanford, CA, USA*
[c] *Benaroya Research Institute, Virginia Mason, Seattle, WA 98101, USA*

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**Abstract** In the present study, we developed a HLA class II tetramer-based detection system utilizing DRB1*0402 tetramers loaded with recently identified immunodominant peptides of desmoglein 3 (Dsg3), the major autoantigen of pemphigus vulgaris (PV). Initial experiments demonstrated staining of a Dsg3-reactive T cell hybridoma which was derived from HLA-DR0402-transgenic mice with loaded PE-labeled DRβ1*0402 tetramers. However, staining of autoreactive T cell clones (TCC) derived from PV patients resulted only in positive staining by addition of exogenous peptides to the staining reactions. There was a dose-dependent specific binding of TCC to the tetramers with the agonistic Dsg3 peptide which was not altered by exogenous unrelated Dsg3 peptide. Noteworthy, the TCC did not stain with HLA-DR4 tetramers complexed with unrelated Dsg3 peptides. The findings of this study suggest that HLA class II tetramers may provide a highly specific approach to monitor ex vivo the T cellular autoimmune response against Dsg3 in patients with PV.


## Introduction

Pemphigus vulgaris (PV) is an autoimmune bullous disease of the skin caused by autoantibodies (auto-Ab) against desmoglein 3 (Dsg3), a component of the desmosomal adhesion complex of epidermal keratinocytes, leading to loss of adhesion between keratinocytes [1]. Current concepts strongly suggest that autoreactive CD4+ T helper cells play a crucial role in the initiation and perpetuation of both Ab- and cell-mediated autoimmune diseases [1]. Peripheral CD4+ T cell responses, and occasionally CD8+ T cell responses, to the ectodomain of Dsg3 were identified in PV patients by several independent investigators [2–4]. However, their phenotype, cytokine profile, immunogenetic restriction and epitope specificity varied. Both Dsg3-reactive Th1 [2] and Th2 [4] cells were identified that recognized portions of the extracellular domain of Dsg3 in the context with PV-associated HLA class II alleles. By ELISPOT and MACS cytokine secretion assay, Dsg3-reactive Th cells were detected at similar frequencies in acute onset, chronic active and remittent PV [5,6].

*Abbreviations:* Dsg3, desmoglein 3; MACS, magnetic cell sorting; PV, pemphigus vulgaris; TCC, T cell clones.

* Corresponding author. Fax: +49 6421 28 65729.
*E-mail addresses:* veldman@med.uni-marburg.de (C. Veldman), hertl@med.uni-marburg.de (M. Hertl).

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**Table 1** Epitope specificity of three desmoglein 3 (Dsg3)-responsive human Th2 cell clones and a murine T cell hybridoma

| TCC | Co4–10 [a] | Co4–28 [a] | P2–3 [a] | T cell hybridoma [b] |
|---|---|---|---|---|
| No Ag | 380 ± 28 | 423 ± 31 | 300 ± 17 | 108 ± 29.7 |
| Dsg1 | 338 ± 36 | 431 ± 39 | 294 ± 9 | 137.5 ± 37.4 |
| Dsg3 | 1454 ± 39 | 1222 ± 61 | 369 ± 12 | 340.5 ± 0.7 |
| DG3(96–112) | 1118 ± 33 | 1907 ± 189 | 289 ± 23 | nd |
| DG3(250–266) | 354 ± 28 | 432 ± 49 | 1618 ± 25 | nd |
| DG3(505–521) | nd | nd | nd | 2724 ± 107.5 |
| PHA | 4865 ± 359 | 5407 ± 202 | 8627 ± 321 | nd |

Nd, not determined; Dsg1, desmoglein 1, Dsg3, desmoglein 3, PHA, phytohemagglutamin.
[a] Specificity was determined by standard [$^3$H]thymidine incorporation assay.
[b] Specificity was determined by murine interleukin 2 immunoreactivity (ELISA).

Both autoreactive Th1 and Th2 cells may be involved in the regulation of the production of pathogenic auto-Ab by B cells in PV since sera of patients with PV contain Th1-regulated IgG1 and Th2-regulated IgG4 auto-Ab directed against Dsg3 [7,8]. Noteworthy, healthy carriers of the PV-associated HLA class II alleles, DRB1*0402 and DQB1*0503, were found to exhibit Dsg3-reactive Th1 cell responses, while healthy carriers of other HLA class II alleles did not [2,5]. In addition, T cell recognition of Dsg3 was restricted by HLA-DRB1*0402 and DQB1*0503 both in PV patients and Dsg3-responsive healthy donors. These observations strongly suggest that (1) there is a predominance of Dsg3-reactive Th2 cells in patients with PV; (2) specific HLA class II alleles are critical for T cell recognition of Dsg3 in PV and health; and (3) Ab production is associated with both Th1 and Th2 cells.

Epitopes of Dsg3 that are recognized by autoreactive CD4$^+$ T cells have been mainly identified utilizing long-term T cell clones [9,10]. Wucherpfennig et al. proposed several candidate peptides of Dsg3 based on their potential anchor motifs with DR$\beta$1*0402 [3]. Three peptides of the extracellular domain of Dsg3 induced a proliferative in vitro response of peripheral lymphocytes from PV patients. Our group extended these findings by the identification of additional epitopes [9–11] which are located within the NH2-terminus of the Dsg3 ectodomain, a region known to harbor the major Ab epitopes [12]. All of the identified Dsg3 epitopes share common anchor residues at relative positions 1, 4 and 6 which were previously identified to be potential HLA class II anchor motifs of DR$\beta$1*0402 [3,9] and carry a positive charge at the position 4 which is critical for binding to the negatively charged P4 pockets of DR$\beta$1*0402 (DR$\beta$70 and 71) and DQB1*0503 (DQ$\beta$57) [10].

The goal of this study was to develop a DR$\beta$1*0402-Dsg3 peptide tetramer system which should be a highly specific tool to detect and quantitate ex vivo functionally relevant, autoreactive T cells in PV. Utilizing this detection system, we were able to directly and specifically detect human Dsg3-peptide-specific T cells. Despite the presumably low affinity of the Dsg3-reactive T cells for the employed tetramers, almost 50% of the Dsg3-reactive T cells were detected using this approach. These findings demonstrate that HLA class II-Dsg3 peptide tetramers may principally represent a highly specific tool to monitor ex vivo autoaggressive T cells in PV.

## Materials and methods

### Proliferative T cell assays

Dsg3-specific T cell clones that were recently generated and characterized [5,10] (Table 1) were cultured at $5\times10^5$ cells with $5\times10^5$ x-irradiated human PBMC (50 Gy) as APC in a final volume of 200 $\mu$l with RPMI 1640 and 10% PHS in 96-well round-bottom microtiter plates (BD-Falcon, Heidelberg, Germany) for 48 h at 37°C. The T cell cultures were either stimulated with 10 $\mu$g/ml Dsg3 or equimolar concentrations of Dsg3 peptides. For the final 18 h, 0.6 $\mu$Ci [$^3$H]thymidine (Dupont, Mechelen, Belgium) was added to each culture. T cell proliferation was then counted in a cell harvester (BD, Heidelberg, Germany) and expressed as a stimulation index (SI), representing the ratio of [$^3$H]thymidine uptake (cpm) in cultures with

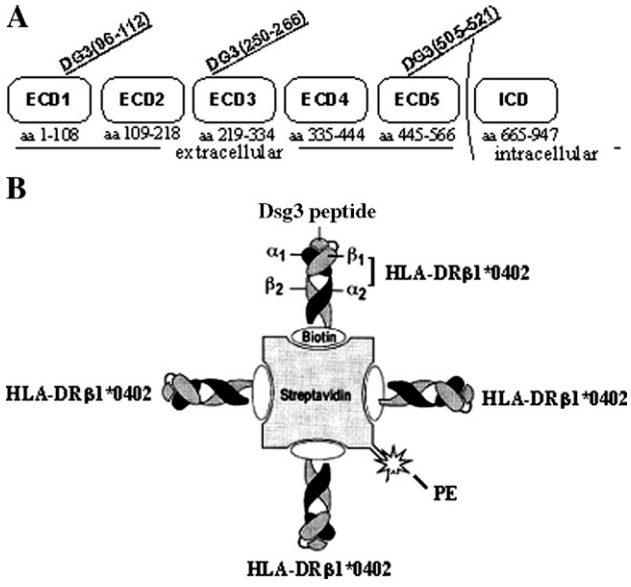


**Figure 1** Components of the DR$\beta$1*0402-peptide tetramers. The three T cell epitopes of Dsg3 which were coupled with the DR$\beta$1*0402 tetramers are located in the extracellular domains (EC) 1, EC3 and EC5 of Dsg3, respectively (A). Shown is the structure of the employed DR$\beta$1*0402-peptide tetramers (B).

antigen and cultures without antigen. An SI >3 was considered to show a significant proliferation. For the production of Dsg3 protein, recombinant PVhis baculovirus was amplified in SF21 insect cells as described previously [2,8]. Recombinant Dsg1, expressed and purified by the same procedure as Dsg3, was used as a control antigen to exclude the possibility that minor contaminants in the antigen preparation induced T cell proliferative responses. T cell peptides were synthesized using Fmoc chemistry (Institute of Biochemistry, University of Erlangen, Germany) resulting in a purity of at least 95%. Lyophilized peptides were reconstituted in $H_2O$ and 0.2% acetic acid at 2 mg/ml and stored at −80°C.

## Tetramer synthesis

Biotinylated DRβ1*0402-soluble molecules were purified as described previously for DRβ1*0401-soluble molecules [13]. In brief, the cDNAs of DRA1*0101 and DRB1*0402 in the Schneider expression vectors pRmHa-3, together with the plasmid pUChsneo (gift from M. McKeown, Salk Institute, San Diego, California, USA), which carries the neomycin resistance marker, were cotransfected into Schneider cells S-2 (gift from D. Zaller, Merck Research Laboratories, Rahway, New Jersey, USA) by standard calcium phosphate transfection techniques. Cells were selected with G418 at 2 mg/ml and were expanded and grown to a density of $10^7$ cells/ml. $CuSO_4$ was added at a concentration of 1 mM to induce the production of soluble HLA class II molecules. The DRβ1*0402 molecules were purified by affinity chromatography using L243 columns [13].

The DRβ1*0402 protein was biotinylated using the Bir A enzyme according to the manufacturer's conditions (Avidity, Denver, Colorado, USA), and excess biotin was removed by dialysis. The biotinylated DRβ1*0402 molecules were then loaded with the Dsg3 peptides by incubation for 72 h at 37°C with 10-fold molar excess of peptides in 100 mM $NaPO_4$, pH 5.5, and 0.2% *n*-octyl-D-glucopyranoside. The Dsg3 peptides employed in this study were DG3(96–112) PFGIFVVDKNTGDINIT, DG3(250–266) QCECNIKVKDVNDNFPM and DG3(505–521) SSPSVVVSARTLNNRYT which represented distinct regions of the Dsg3 ectodomain (Fig. 1A). The peptide loaded HLA-DRβ1*0402 tetramers were then incubated overnight at RT with phycoerythrin (PE)–streptavidin (BioSource International, Camarillo, California, USA) at a 8:1 molar ratio to allow the formation of tetrameric class II peptide complexes.

## Tetramer staining

Three Dsg3-responsive TCC and a Dsg3-reactive murine T cell hybridoma (Table 1) were stained with DRβ1*0402-Dsg3 peptide tetramer (diluted at 1:50) and exogenously added Dsg3 peptides in RPMI 1640 (Gibco) with 10% PHS (Gibco) in 24-well plates (BD-Falcon, Heidelberg, Germany) at RT for 1 h. Finally, the derived T cells were washed and analyzed by flow cytometry (FACS ScanTM and CELLQuestTM Software, BD-Biosciences, Heidelberg, Germany).

## HLA-DRβ1*0402-transgenic mice

HLA-transgenic mice expressing HLA-DRA*0101/DRB1*0402 and the human CD4 co-receptor were described previously [14]. These mice were crossed with HLA-DQA1*0301/DQB1*0302 (DQ8)-transgenic mice to obtain HLA-DR0402-DQ8 expressing founder lines. Both the HLA-DR0402- and the HLA-DQ8-transgenic lines lack functional murine MHC class II molecules (I-$A\beta^{-/-}$) [15]. Mice were backcrossed to the DBA/1J background, bred and maintained at the research animal facilities at Stanford University, CA, USA and at Philipps

**Table 2** Reported use of HLA class II tetramers in human diseases

| HLA class II allele | Antigens | Disease | References |
|---|---|---|---|
| DRβ1*0101 | HCV NS3 peptide | Hepatitis C virus infection | [26] |
| | Hemagglutinin | Influenza | [26–29] |
| | CMV pp65 | Cytomegalovirus infection | [26] |
| | HIV p24 Gag | Human immunodeficiency virus infection (HIV) | [26,30] |
| | Tetanus toxoid | Tetanus | [26] |
| DRβ1*0401 | HIV p24 Gag | Human immunodeficiency virus infection (HIV) | [26] |
| | Hemagglutinin | Influenza | [13,27–29,31] |
| | EBV-BHRF1 | Infectious mononucleosis | [32] |
| | OspA | Lyme disease | [33] |
| | HSV-2VP16 | Herpes simplex type 2 infection | [34] |
| | GAD65 | Diabetes mellitus | [19,35,36] |
| | RP CII | Relapsing polychondritis | [37] |
| | NY-ESO-1 | Melanoma | [27] |
| | HSV p61 | Herpes simplex infection | [34] |
| | Lol-p-1 | Gras pollen allergy | [38] |
| DRβ1*0402 | Dsg3 | Pemphigus vulgaris | Present study |
| DRβ1*0404 | HSV p61 | Herpes simplex type 2 infection | [34] |
| | HSV-2 P16 | Herpes simplex type 2 infection | [39] |
| | GAD65 | Diabetes mellitus | [36,40] |
| DQβ1*0201 | Gliadin | Celiac disease | [41] |
| DQβ1*0602 | HSV-2 VP16 | Herpes simplex infection | [42] |

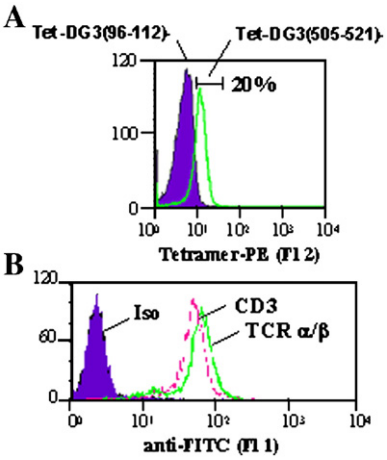


**Figure 2** DRβ1*0402-peptide tetramer staining of a DG3 (505–521)-specific murine T cell hybridoma. A DG3(505–521)-responsive T cell hybridoma was generated from a HLA-DRβ1*0402 transgenic mouse after immunization with human Dsg3. Upon incubation with the DRβ1*0402-DG3(505–521) tetramer, more than 20% of the T cells stained positive, while there was no specific staining (<3%) with the irrelevant DRβ1*0402-DG3(96–112) tetramer (A). The Dsg3-reactive T cell hybridoma was CD3/TCRα/β positive suggesting that not low TCR expression was the reason for the rather weak tetramer staining (B).

University, Center for Biomedical Research (BMFZ), Marburg, Germany.

## Dsg3-specific T cell hybridoma

HLA-DR0402-DQ8-/human CD4-transgenic, I-Aβ$^{-/-}$ mice were immunized with 50 μg of purified recombinant human Dsg3 protein emulsified in complete Freund's adjuvant (CFA, Sigma) in the hind footpads and base of the tail. Ten days later, the draining popliteal and inguinal lymph nodes were removed, and lymph node T cells were restimulated in vitro with Dsg3 (20 μg/ml) for 72 h. Activated T cells were expanded with murine IL-2 (10 U/ml) for another 24 h, and T cell hybridomas were generated by fusing T cells from lymph nodes with the TCR α/β$^{-/-}$ variant of the BW 5147 thymoma cell line as described previously [14]. Growing T cell hybridomas ($5 \times 10^4$) were incubated with $2 \times 10^5$ x-irradiated splenocytes as APC and the respective antigens and screened for antigen-specific IL-2 secretion after 16–18 h using a horseradish peroxidase-based murine IL-2-ELISA (BD-Pharmingen, Heidelberg). All Dsg3-reactive T cell hybridomas were tested with pools of overlapping synthetic Dsg3-peptides (Chiron Mimotopes, San Diego/CA, USA) spanning the entire Dsg3 ectodomain to define their epitope specificity [16].

# Results

## Staining of a Dsg3-reactive murine T cell hybridoma with a DRβ1*0402-Dsg3 peptide tetramer

A Dsg3-reactive mouse T cell hybridoma showed a proliferative response to DG3(505–521) as determined by IL-2 secretion (Table 2) and was restricted by HLA-DRβ1*0402 [16]. More than 20% of the T hybridoma cells stained with the DG3(505–521) tetramer, while the negative control DG3 tetramer (Tet-DG3(250–266)-PE) did not specifically stain the T cell hybridoma cells (Fig. 2A). To exclude low expression of the TCR complex on the cell surface as an explanation of the incomplete staining of the T cell hybridoma cells with the DRβ1*0402-Dsg3 peptide tetramer, CD3 and TCRα/β expression was investigated by FACS. As shown in Fig. 2B, more than 96% of the T cell hybridoma cells expressed the CD3/TCR complex on their cell surface.

## Staining of Dsg3-responsive TCC with Dsg3 peptide-DRβ1*0402 tetramers

Three Dsg3-specific Th2 clones which recognized Dsg3 peptides DG3(96–112) (TCC Co4–10 and Co4–28) or DG3 (250–266) (TCC P2–3) were used in this study (Table 1). Initial staining indicated that tetramer binding was negative. However, addition of exogenous peptide to the staining reaction mixtures led to positive staining. The extent of the staining reaction was dependent on the concentration of exogenously added peptide and reached a maximum of 47.3% at 50 μg/ml of the relevant Dsg3 peptide (Fig. 3A). Tetramer binding was specific since the TCC did not stain with a

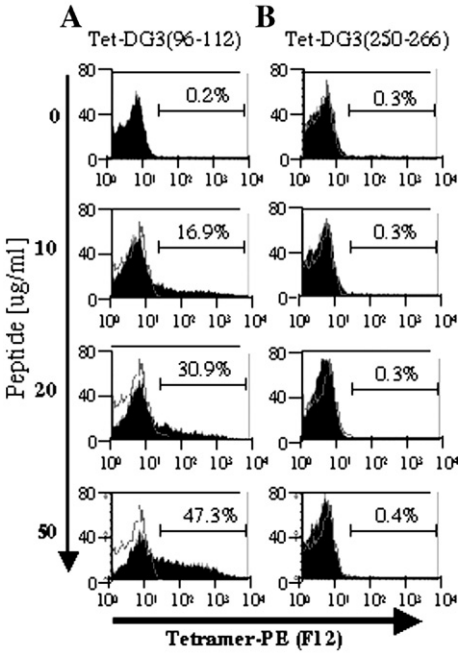


**Figure 3** Specific staining of a DG3(96–112)-specific human CD4$^+$ T cell clone with a HLA class II tetramer. The DG3(96–112)-specific CD4$^+$ T cell clone Co4–28 showed a dose-dependent staining with the DRβ1*0402-DG3(96–112) tetramer, reaching a maximum of 47.3% positive T cells at 50 μg of the agonistic Dsg3 peptide, DG3(96–112) (A). In contrast, incubation with the irrelevant DRβ1*0402-peptide tetramer, Tet-DG3(250–266), did not lead to a specific staining of the TCC Co4–28 (B). Staining with specific tetramer without addition of exogenous peptide, shown as non-shaded curves, served as control.

DRβ1*0402 tetramer loaded with an irrelevant Dsg3 peptide (Fig. 3B).

## Specificity of Dsg3 peptide-HLA class II tetramer binding

Since the characterized T cell epitopes of Dsg3 show highly conserved anchor motifs at relative positions p1, p4 and p6 for the HLA-DRβ1*0402 molecule [10], specific binding of the TCC to the DRβ1*0402 tetramers loaded with their relevant Dsg3 peptide had to be confirmed with irrelevant Dsg3 peptides. TCC were incubated together with their specific DRβ1*0402-peptide tetramers and varying concentrations of exogenously added specific or irrelevant Dsg3 peptide. There was a dose-dependent specific binding of the TCC with the HLA-DRβ1*0402 tetramer complexed with their nominal Dsg3 peptides reaching a maximum of 39.5 to 44.7% of positive T cells at a concentration of 50 μg/ml of the relevant Dsg3 peptide (Fig. 4A–B). After addition of increasing concentrations of an unrelated Dsg3 peptide, specific tetramer staining was not inhibited (Fig. 4A–B). Tetramers loaded with irrelevant Dsg3 peptides at high concentrations led to a background staining of the TCC ranging from 4.1 to 63% (Fig. 4A–B).

## Discussion

While the application of human class I tetramers to study self antigens has been most extensively developed in studies of tumor antigens [17], the use of human HLA class II multimers

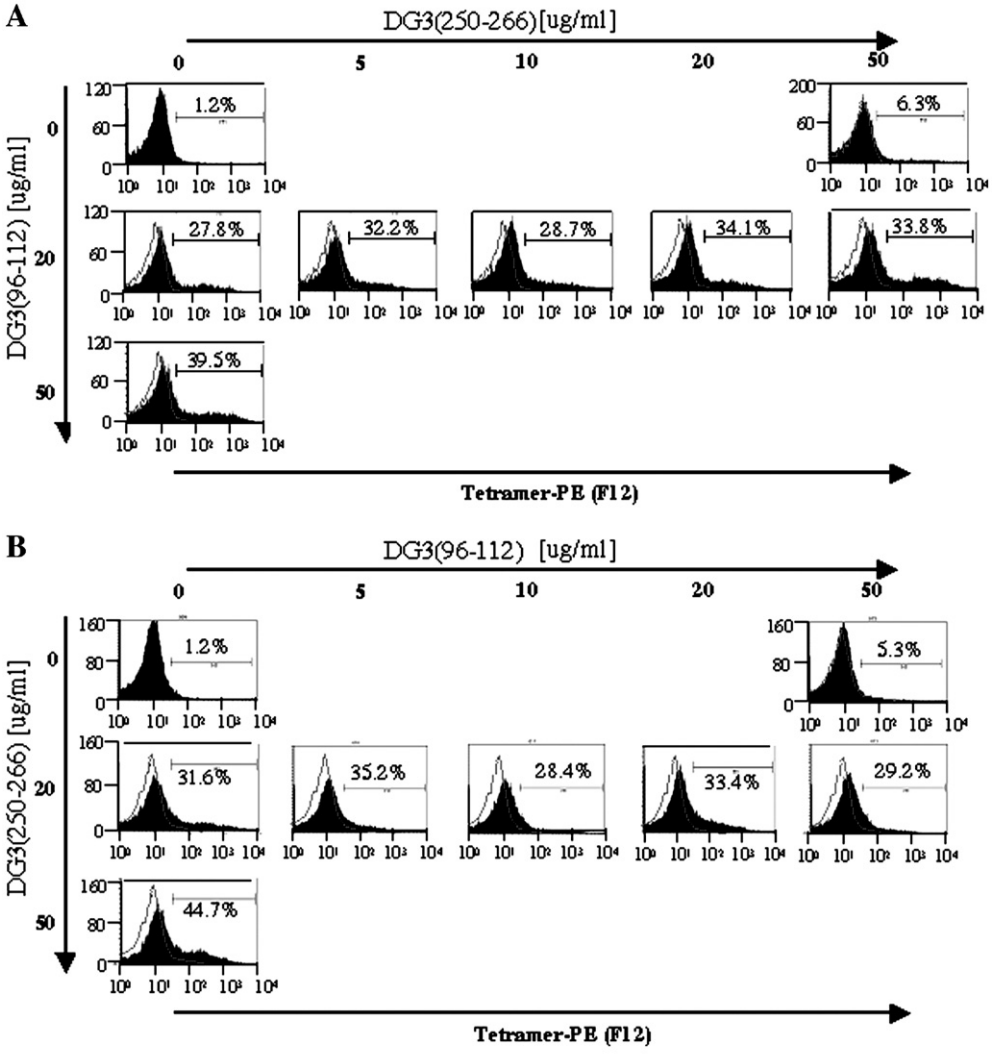


**Figure 4** Specificity of DRβ1*0402-(desmoglein) Dsg3 peptide tetramer staining of human autoreactive T cell clones (TCC). The DG3 (96–112)-specific TCC Co4–10 showed a dose-dependent staining with the DRβ1*0402-DG3(96–112) tetramer with a maximum of 39.5% positive T cells at 50 μg/ml DG3(96–112) (A). This staining intensity was not significantly altered when increasing doses (5–50 μg/ml) of an irrelevant Dsg3 peptide, i.e. DG3(250–266) were added to the staining mix (A). Similarly, the DG3(250–266)-specific TCC P2–3 showed a dose-dependent staining with the DRβ1*0402-DG3(250–266) tetramer which reached a maximum of 44.7% at 50 μg/ml of DG3(250–266) (B). Upon adding the irrelevant Dsg3 peptide, DG3(96–112), ranging from 5 to 50 μg/ml, the staining intensity of P2–3 with the DRβ1*0402-DG3(250–266) tetramer was not significantly altered (B). Staining with specific tetramer without addition of exogenous peptide, shown as non-shaded curves, served as control.

concentrated primarily on the detection and monitoring of human T cell responses to infectious antigens, for which the $CD4^+$ T cell response is strong and epitope specificity is fairly predictable. A panel of so far described HLA class II tetramer complexes with peptides of various (auto-) antigens is shown in Table 2.

T cell receptors (TCR) reactive to self antigens, including autoantigens of human autoimmune disorders and tumors, may have lower affinity for the HLA/peptide complexes compared to TCR which are reactive to foreign antigens [18]. Autoreactive T cells are expected to express low affinity TCR to escape thymic negative selection. T cells that are directed against autoantigens were shown not to bind to their specific tetramers [19]. A higher degree of cross-linking of the HLA molecules leading to tetrameric HLA complexes should increase the avidity between the TCR and the HLA class II molecule. Different approaches for oligomerization of HLA molecules have been attempted. Chemical cross-linking of the HLA molecule with synthetic cross linkers is one approach [20], incorporation of HLA molecules into liposomes another [21]. The use of chimeric HLA-Ig has also been attempted. However, use of these reagents to stain low affinity T cells has not been successfully employed [22].

In this study, we developed a class II tetramer-based detection system using HLA-DR$\beta$1*0402 tetramers loaded with previously identified T cell epitopes of Dsg3 to detect ex vivo human Dsg3-reactive T cells in patients with PV. Using this approach, we were able to detect autoreactive T cells with the tetramer reagents. T cell staining with the HLA class II tetramers was highly specific leading to the detection of functionally relevant effector Th cells. We observed that addition of specific peptide exogenously was essential for staining of low avidity T cells. The tetramers used in this study do not contain a covalently bound peptide, but the peptide is loaded into the "empty" tetramer construct. So, we speculate that addition of exogenous peptide to the staining reaction enhances the stability of the tri-molecular MHC-peptide–TCR interaction by increasing the "on rate" of peptide binding to the tetramer complex, leading to positive staining. The findings of this study provide the basis for a tetramer-based approach to monitor ex vivo autoreactive T cells as a novel and most probably more sensitive cellular immune parameter in patients with PV.

In addition to the difficulty in detection of low avidity T cells, the application of HLA class II tetramer technology in autoimmune disorders may be impeded by the low number of autoantigen-specific $CD4^+$ T cells present in the peripheral blood and may preclude direct staining of antigen-specific T cells with tetramers ex vivo.

Previously, frequency analyses of autoaggressive T cells in PV had been performed either by cell culture-based T cell proliferative assays or by enzyme-linked immunospot (ELISPOT) assays which measures the secretion of distinct Th cytokines upon ex vivo stimulation by Dsg3 [5,6,10]. By ELISPOT assay, autoreactive Th1/Th2 cells were detectable at similar frequencies ($4.7 \pm 2.4$ for Th1 cells and $3.0 \pm 0.4$ Th2 cells per $10^5$ PBMC) in acute onset PV [6]. To confirm the ELISPOT-derived data, a recent study sought to quantitate peripheral Dsg3-responsive Th1 and Th2 cells in PV patients by MACS cytokine secretion assay [5]. Both Dsg3-autoreactive Th1 and Th2 cells were isolated from patients with acute onset, chronic active and remittent PV leading to the detection of autoreactive Th1 and Th2 frequencies that were comparable to those obtained by ELISPOT analysis. The titers of serum auto-Ab against Dsg3 correlated well with the ratio of autoreactive Th1/Th2 cells, suggesting that both Th1 and Th2 cells may be critically involved in the regulation of auto-Ab production. These functional assays are solely dependent on the responsiveness of Dsg3-reactive T cells to Dsg3 stimulation. The results may be significantly influenced by the experimental conditions, including the ex vivo selection of autoreactive T cells, the relationship between T cells and APC, the concentration and purity of the employed antigen(s) and the cell culture conditions. In addition, the aforementioned assays may not equally detect the entire spectrum of autoreactive T cells with differential epitope specificity depending on their proliferative capacity in vitro [10].

Despite the limitations of the present assay, the use of HLA class II tetramers has great potential to provide a unique approach to specifically monitor ex vivo the activation of distinct subsets of autoaggressive effector T cells in PV. Clinical examples for such an approach are recent studies utilizing HLA class I tetramers to study T cell responses against melanoma antigens in patients with malignant melanoma and HIV-derived antigens in patients with HIV infection [23–25]. The use of HLA class II tetramers to study the pathogenesis PV, a classical HLA-linked autoimmune disease, and monitor the changes of the autoreactive T cells during the clinical course will be a major challenge in the near future.

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