

# Immunomodulatory potential of heteroclitic analogs of the dominant T-cell epitope of lipocalin allergen Bos d 2 on specific T cells

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## Abstract

**Peptide-based allergen immunotherapy is a novel alternative for conventional allergen immunotherapy. Here, we have characterized the immunomodulatory potential of heteroclitic peptide analogs of the immunodominant epitope of lipocalin allergen Bos d 2 on specific human T-cell clones. The TCR affinity of Bos d 2-specific T-cell clones for the natural peptide ligand and its heteroclitic analogs was assessed with fluorescent-labeled MHC class II tetramers. The activation and cytokine production of the clones were analyzed upon stimulation with the different ligands. Moreover, the capacity of the heteroclitic analogs to induce hyporesponsiveness and cell death was examined. The T-cell clones F1-9 and K3-2 bound MHC class II tetramers loaded with the heteroclitic peptide analogs of the immunodominant epitope of Bos d 2 with increased affinity. At similar peptide concentrations, stimulation of the clones with the heteroclitic analogs favored increased IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-5 ratios in comparison with stimulation with the natural peptide ligand. Moreover, the T-cell clones stimulated with the heteroclitic analogs exhibited an increased susceptibility to cell death or hyporesponsiveness upon re-stimulation. Our results suggest that heteroclitic analogs of a T-cell epitope of an allergen may enhance the efficacy of peptide-based allergen immunotherapy.**

## Introduction

The increased incidence of allergic diseases is a widely recognized health problem in industrialized countries. To fight against allergy, effective and safe forms of therapy directed against the underlying immune mechanisms are urgently needed. Since the role of T cells, particularly T<sub>H</sub>2-type effector cells, in the development of IgE-mediated allergic diseases is well established (1), the targeted modulation of their function by allergen immunotherapy is an attractive therapeutic alternative. Conventional allergen immunotherapy, often referred to as specific immunotherapy (SIT), involves the administration of increasing doses of an allergen extract by subcutaneous injections (2). The major drawbacks of this treatment are the risk of systemic reactions resulting from the cross-linking of IgE on mast cells and basophils and the inconsistent quality of the extracts used. These problems may be circumvented by using short peptides containing the immunodominant T-cell epitopes

of an allergen (3). Recent clinical trials have demonstrated the potential of peptide-based allergen immunotherapy in treating allergy (4–7).

Altered peptide ligands (APLs) are peptides containing amino acid substitutions as compared with the natural peptide. They can induce both quantitatively and qualitatively distinct signals in the responding T cell (8, 9). APLs have been used successfully for treatment in the murine models of autoimmune diseases and cancer (10–14). Some studies suggest that they also have immunomodulatory potential on allergen-specific T cells (15–18).

We have previously identified heteroclitic APLs for the T-cell clones specific for the immunodominant epitope of Bos d 2, a lipocalin allergen (19, 20). The two single-amino acid variants, pN135D and pQ133K, were able to stimulate the clones F1-9 and K3-2, respectively, at 10- to 100-fold lower peptide

## 2 Immunomodulatory potential of heteroclitic analogs

concentrations than the natural ligand. In the present study, we are able to show directly by MHC class II tetramer staining that the TCR affinity of the clones for their natural ligand is sub-optimal. We suggest that heteroclitic analogs have immunomodulatory potential since stimulation of the T-cell clones with the analogs favored  $T_H1$ -biased cytokine production *in vitro*. Moreover, upon stimulation with the analogs, the clone F1-9 exhibited an increased susceptibility to cell death and the clone K3-2 became hyporesponsive to re-stimulation with the natural ligand. Our results suggest that heteroclitic peptide ligands may open new prospects for peptide-based allergen immunotherapy.

### Methods

#### *Cells and synthetic peptides*

T-cell clones F1-9, K3-2, K9-10 and T144 specific for the immunodominant epitope of Bos d 2, present in peptide p127–142, were isolated and propagated as described previously (19). The cells were cultured in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 2 mM L-glutamine, 20  $\mu$ M 2-mercapto-ethanol, sodium pyruvate (BioWhittaker), non-essential amino acids (BioWhittaker), 100 IU ml<sup>-1</sup> penicillin, 100  $\mu$ g ml<sup>-1</sup> streptomycin, 10 mM HEPES (BioWhittaker) and 5% inactivated human AB serum (Finnish Red Cross, Helsinki, Finland). The clones F1-9 and K3-2 have been shown to be restricted by DRB1\*0401 (20). Using the same approach as previously, clone K9-10 was also observed to be restricted by DRB1\*0401 and the clone T144 by DR2 (DRB1\*1501 or DRB5\*0101, data not shown). The clonality of the T-cell clones was proven by PCR using a panel of TCR subfamily-specific primers (20). The TCR usage of the clones was V $\alpha$ 2/V $\beta$ 7.2 for F1-9, V $\beta$ 13.1 for K3-2, V $\beta$ 14.1 for K9-10 and V $\alpha$ 14.1/V $\beta$ 13.1 for T144. The V $\alpha$  usage of the clones K3-2 and K9-10 could not be identified with the primer panel used (data not shown). The EBV-transformed B cell lines BOLETH (HLA-DRB1\*0401) and SCHU (DR2) were used as antigen-presenting cells (APCs) in all assays. The Bos d 2 peptide p127–142 (ELEKYQQLN-SERGVPN) and its single-amino acid variants (pN135D and pQ133K) were synthesized and verified as described previously (20).

#### *HLA class II tetramers*

The generation of DRA1\*0101/DRB1\*0401-soluble class II molecules and the procedure used for peptide loading have been described earlier (21). The peptides described above were used to load DRB1\*0401 molecules to generate DRB1\*0401/peptide tetramers. The peptide 64–78 (QVFQVSHSFPHPYD) of prostate-specific antigen (PSA) was used to generate DRB1\*0401/PSA64-78 as a control tetramer for staining. The staining was performed by incubating  $5 \times 10^4$  T cells with different concentrations of the PE-labeled tetramers in culture medium for 2 h at 37°C. Anti-CD4-FITC (BD Biosciences, San Jose, CA, USA) was added and the cells were incubated for an additional 20 min at +4°C. The cells were then washed twice and analyzed with a FACScan flow cytometer (BD, Mountain View, CA, USA). Staining with FITC-labeled anti-TCR $\alpha\beta$  (BD) was performed separately and the dose–response curve of median fluorescence intensity (MFI) tetramer staining

was normalized to that of the TCR staining, as described previously (22).

#### *Functional assays*

T-cell proliferation assays were set up in triplicates in 96-well round-bottomed microtiter plates (Corning Inc., Corning, NY, USA) with  $2.5 \times 10^4$  T cells,  $5 \times 10^4$   $\gamma$ -irradiated (6000 rad) APCs and peptides at different concentrations. After 72 h of incubation, [<sup>3</sup>H]thymidine was added (1  $\mu$ Ci per well; Amersham Pharmacia Biotech, Little Chalfont, UK), and after an additional 16 h, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland). Radioactivity was measured by scintillation counting (Wallac Micro Beta 1450) and the results were expressed as counts per minute.

For cytokine analyses,  $5 \times 10^5$  T cells and  $5 \times 10^5$  irradiated APCs were incubated with different concentrations of the peptides in a total volume of 1 ml. After 24 h, culture supernatants were collected and stored at –70°C until analyzed. For the measurement of IL-5 levels in the supernatants, the LyH7.B13 cell line was used as described previously (19). The concentrations of IFN- $\gamma$  and IL-4 were measured in duplicate using commercial ELISA kits (DuoSet, R&D Systems, Minneapolis, MN, USA) with AMDEX amplification (Amersham Pharmacia Biotech), as described by the manufacturers.

For analyzing the TCR and CD25 expressions by the T-cell clones,  $5 \times 10^4$  T cells were stimulated with different concentrations of the peptides with  $5 \times 10^4$  irradiated APCs in 96-well round-bottomed microtiter plates for 20 h. The cells were then stained with FITC-labeled anti-TCR $\alpha\beta$ , PE-labeled anti-CD25 and PerCP-labeled anti-CD4 antibodies (all from BD) for 20 min at +4°C, washed twice and analyzed with the flow cytometer. The results are expressed as MFI.

#### *T-cell survival assays*

For monitoring the fate of T cells after stimulation with the different peptides,  $2.5 \times 10^4$  T cells and  $5 \times 10^4$  irradiated BOLETH cells were incubated with or without the peptides (5  $\mu$ M) in triplicate in 96-well round-bottomed microtiter plates. Starting on day 1 and every 3 days thereafter, half of the culture medium was replaced with fresh medium supplemented with 10 IU ml<sup>-1</sup> of recombinant IL-2 (Strathmann Biotech, Hannover, Germany). At different time points, the cells were harvested from the plates, and triplicate samples were pooled. The number of live cells was determined as described previously (23), with some modifications. In brief, the cells were stained with PE-labeled anti-CD4-PE and FITC-labeled anti-TCR $\alpha\beta$  for 20 min at +4°C and washed twice. The cells were then incubated for an additional 10 min at room temperature with the vital dye 7-amino-actinomycin D (7-AAD; BD Pharmingen). After this period, the cell samples were suspended in 200  $\mu$ l of PBS and analyzed with the FACScan flow cytometer. Events were counted for 60 s to allow comparative analysis between samples. The number of CD4-positive 7-AAD-negative cells was calculated, and the TCR expression was analyzed in this population. In re-stimulation experiments with clone K3-2, viable cells were collected on day 10 by density gradient centrifugation.

## Results

### Activation of *Bos d* 2-specific T-cell clones

In this study, we have examined the functional responses of four *Bos d* 2-specific T-cell clones, F1-9, K3-2, K9-10 and T144, isolated from three different cow-asthmatic subjects (19). As reported previously (20), heteroclitic single-amino acid variants pN135D and pQ133K induced the proliferation of the clones F1-9 and K3-2, respectively, at lower concentrations than the natural peptide ligand p127–142 (Fig. 1). To assess in detail the stimulatory capacity of the natural ligand and its derivatives (Fig. 1), TCR down-regulation, an early

quantitative indicator of T-cell activation (24, 25), was measured. It was observed that p127–142 induced the down-regulation of TCR dose dependently in all the clones (Fig. 1). Interestingly, the heteroclitic analogs could induce the TCR down-regulation of F1-9 and K3-2 at lower peptide concentrations than p127–142 (Fig. 1). Moreover, the extent of TCR down-regulation was greater upon stimulation with the heteroclitic ligands. To confirm the results, the expression of CD25 (IL-2R), which is dependent on the activation status of a T cell (26), was determined. The stimulation of *Bos d* 2-specific T-cell clones with p127–142 led to a dose-dependent up-regulation of CD25 (Fig. 1). As expected, stimulation of the clones F1-9

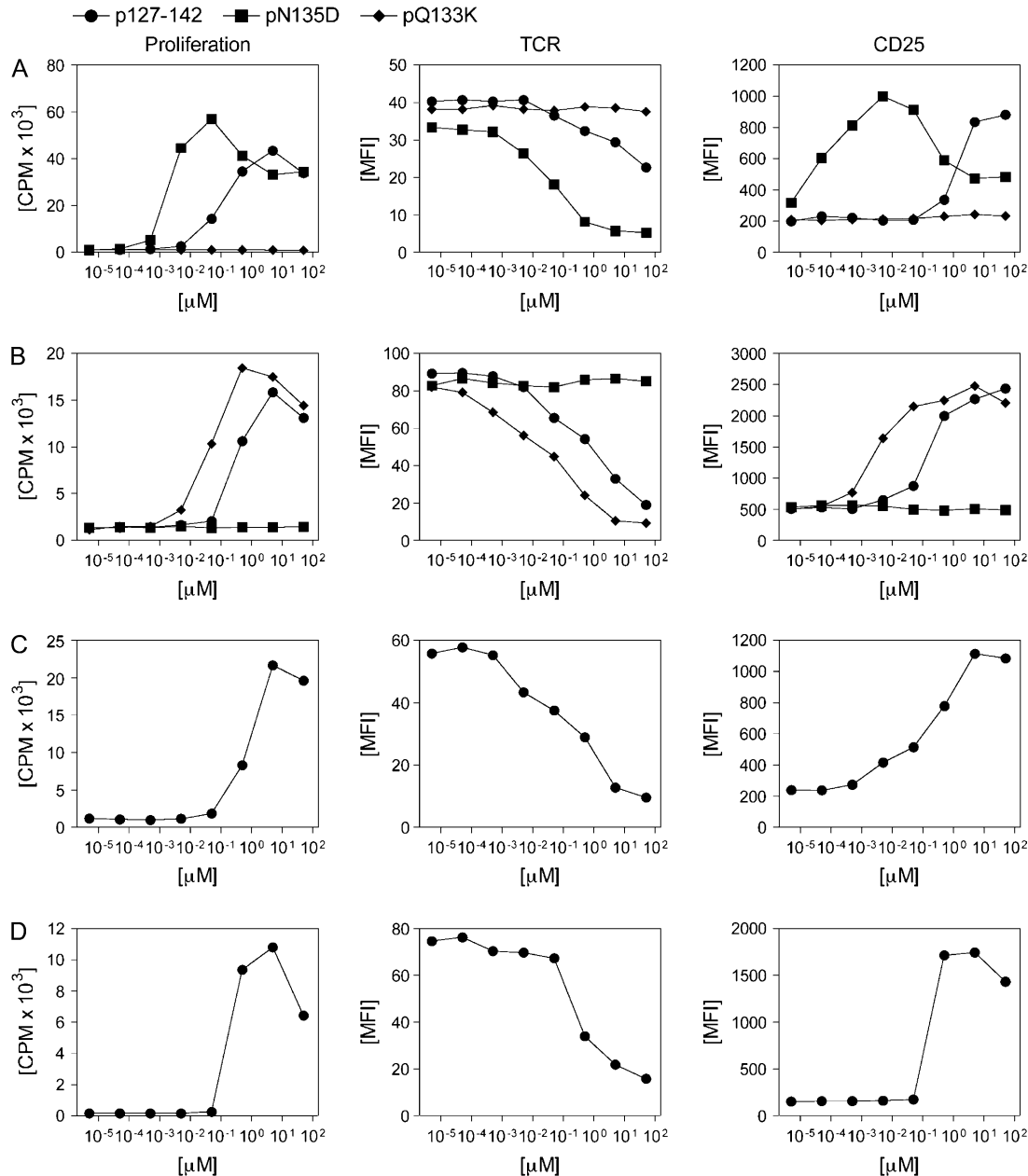


**Fig. 1.** Activation of *Bos d* 2-specific T-cell clones with p127–142 or its single-amino acid variants pN135D and pQ133K. Proliferation, TCR down-regulation and CD25 expression of F1-9 (A), K3-2 (B), K9-10 (C) and T144 (D) at different peptide concentrations are shown. The results are representative of three independent experiments.

#### 4 Immunomodulatory potential of heteroclitic analogs

and K3-2 with the heteroclitic ligands induced the up-regulation of CD25 at lower concentrations than the natural ligand. The maximal expression of CD25 was not, however, higher than that induced by p127–142.

##### *Tetramer binding by Bos d 2-specific T-cell clones*

As the binding of MHC class II tetramers at non-saturating concentrations is a straightforward measure of TCR affinity (22, 27–29), we used fluorescent-labeled DRB1\*0401 tetramers to assess the TCR affinity of the Bos d 2-specific T-cell clones for the natural ligand p127–142 and the heteroclitic peptide analogs pN135D and pQ133K. An example of staining with DRB1\*0401/p127–142 tetramer is shown in Fig. 2(A). To allow comparison between the clones, the binding of the tetramers was measured over a range of concentrations and the results were normalized to the TCR $\alpha\beta$  expression of the clones (Fig. 2B). Clones K3-2 and K9-10 showed approximately similar affinities for DRB1\*0401/p127–

142. Clone F1-9, however, failed to bind DRB1\*0401/p127–142 at any of the concentrations used, suggesting a much weaker TCR affinity for the tetramer. When the tetramers loaded with the heteroclitic analogs were used for staining, clone F1-9 was positive with DRB1\*0401/pN135D while clone K3-2 bound the DRB1\*0401/pQ133K tetramer at a higher affinity than DRB1\*0401/p127–142. These findings point to the increased TCR affinity of F1-9 and K3-2 for the heteroclitic analogs pN135D and pQ133K, respectively. None of the three DRB1\*0401-restricted T-cell clones was stained with the control tetramer. The DR2-restricted clone T144 was also tested, but it was not stained with the DRB1\*0401 tetramers (Fig. 2B).

##### *Cytokine production by the Bos d 2-specific T-cell clones*

All the T-cell clones produced IFN- $\gamma$ , IL-4 and IL-5 upon stimulation with p127–142 (Fig. 3). According to their cytokine production, clones F1-9 and K9-10 can be regarded as T<sub>H</sub>0 like and the clones K3-2 and T144 as T<sub>H</sub>2 like. When clones

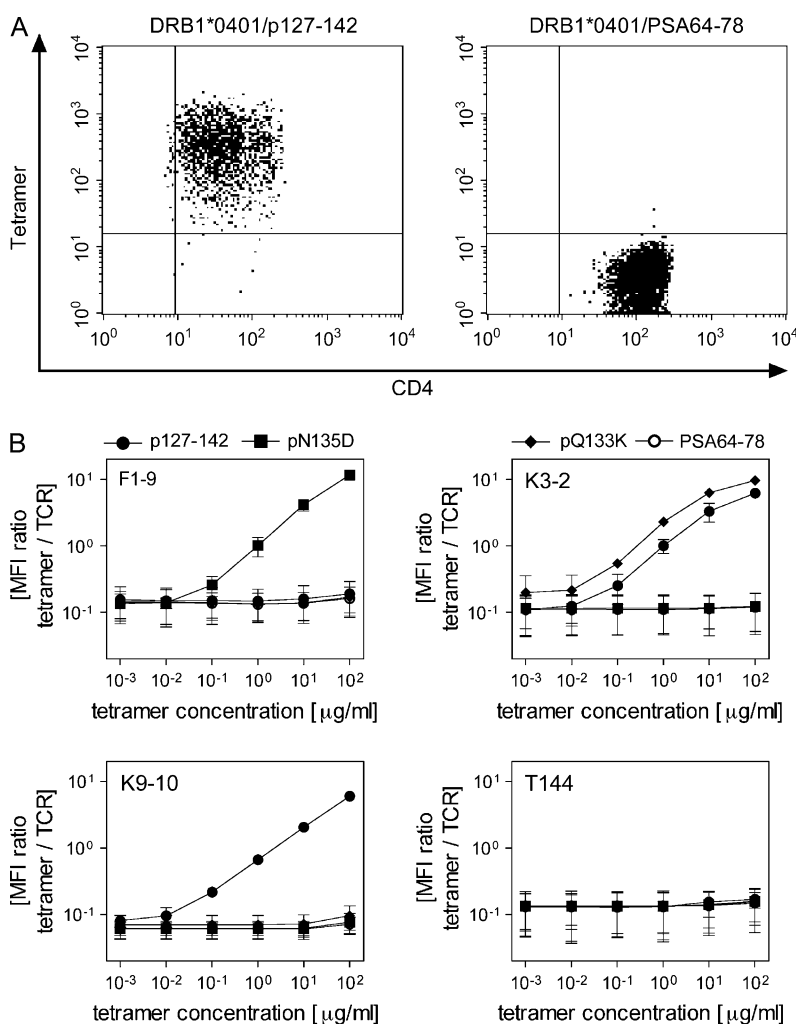


**Fig. 2.** Staining of Bos d 2-specific T-cell clones with DRB1\*0401 tetramers. (A) Staining of clone K3-2 with either 10  $\mu\text{g ml}^{-1}$  of DRB1\*0401/p127–142 (left panel) or the control tetramer DRB1\*0401/PSA64-78 (right panel). (B) Binding profiles of the DRB1\*0401 tetramers on T-cell clones. Median fluorescence (MFI) of tetramer staining was normalized to the median fluorescence of staining with an anti-TCR $\alpha\beta$  mAb. The mean  $\pm$  1 SD of three independent experiments are shown.

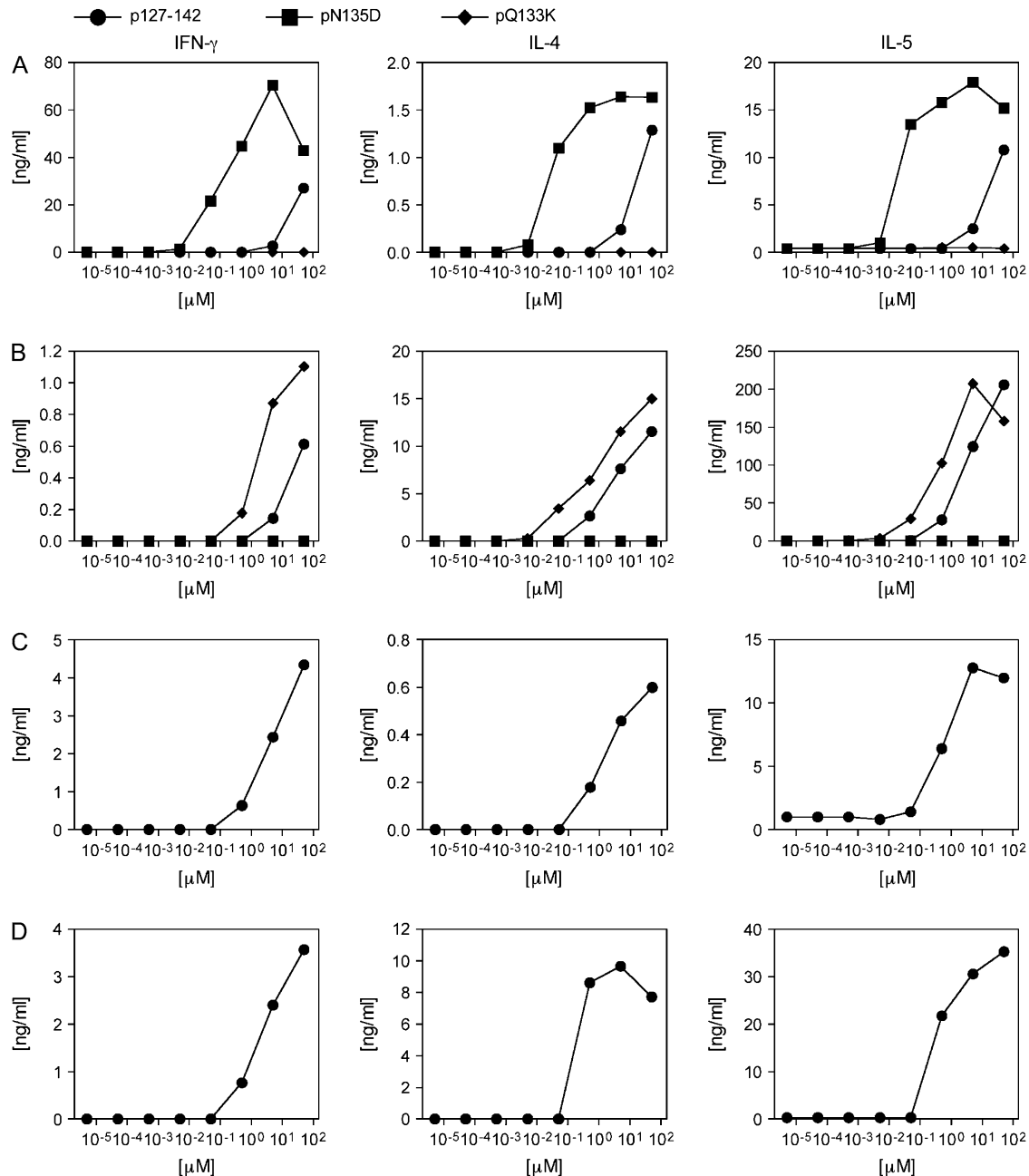


**Fig. 3.** Cytokine production by Bos d 2-specific T-cell clones. The production of IFN- $\gamma$ , IL-4 and IL-5 by F1-9 (A), K3-2 (B), K9-10 (C) and T144 (D) at different peptide concentrations is shown. The results are representative of three independent experiments.

F1-9 and K3-2 were stimulated with their respective heteroclitic peptide ligands, the production of cytokines was detectable at lower concentrations than with p127-142, corresponding to the observed differences in TCR affinity.

Although all the clones produced IFN- $\gamma$  together with IL-4 and IL-5, the production of the T<sub>H</sub>2-type cytokines tended to reach a plateau at lower peptide concentrations than did the production of IFN- $\gamma$  (Fig. 3). This was reflected in the ratios of IFN- $\gamma$ /IL-4 and IFN- $\gamma$ /IL-5, which increased with increasing concentrations of p127-142 with all the clones (Fig. 4). When clones F1-9 and K3-2 were stimulated with the heteroclitic

peptides, the ratios were higher than those observed with the equimolar concentrations of the natural peptide (Fig. 4).

*Stimulation with heteroclitic peptide ligands leads to increased susceptibility to cell death and hyporesponsiveness*

The T-cell clones F1-9 and K3-2 stimulated with the natural ligand p127-142 or the heteroclitic peptide analogs were monitored for 10 days to evaluate the effect of the ligands on T-cell growth and function. When clone F1-9 was stimulated

## 6 Immunomodulatory potential of heteroclitic analogs

with p127-142, only a minimal TCR down-regulation could be observed, while the stimulation of clone K3-2 with the same ligand led to a stronger TCR down-regulation, which lasted for several days but reached the level of the unstimulated control cells by the end of the culture period

(Fig. 5A). In contrast, stimulation of the clones with the heteroclitic ligands resulted in enhanced TCR down-regulation. It was especially distinct for F1-9 at the beginning of the culture period, while for K3-2 it was more obvious at the end of the period. With both clones, the TCR down-regulation

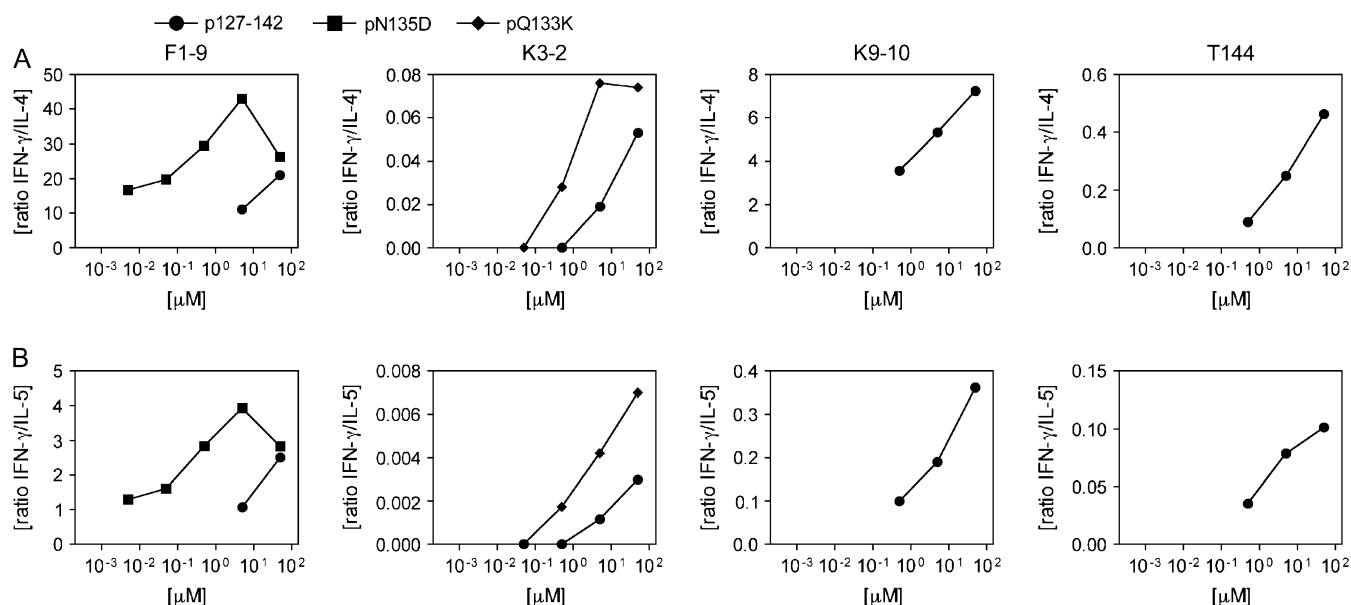


**Fig. 4.** Strong stimulation of Bos d 2-specific T-cell clones leads to elevated T<sub>H</sub>1 to T<sub>H</sub>2 cytokine ratios. Ratios of IFN-γ/IL-4 (A) and IFN-γ/IL-5 (B) at different peptide concentrations were calculated from the data in Fig. 3.

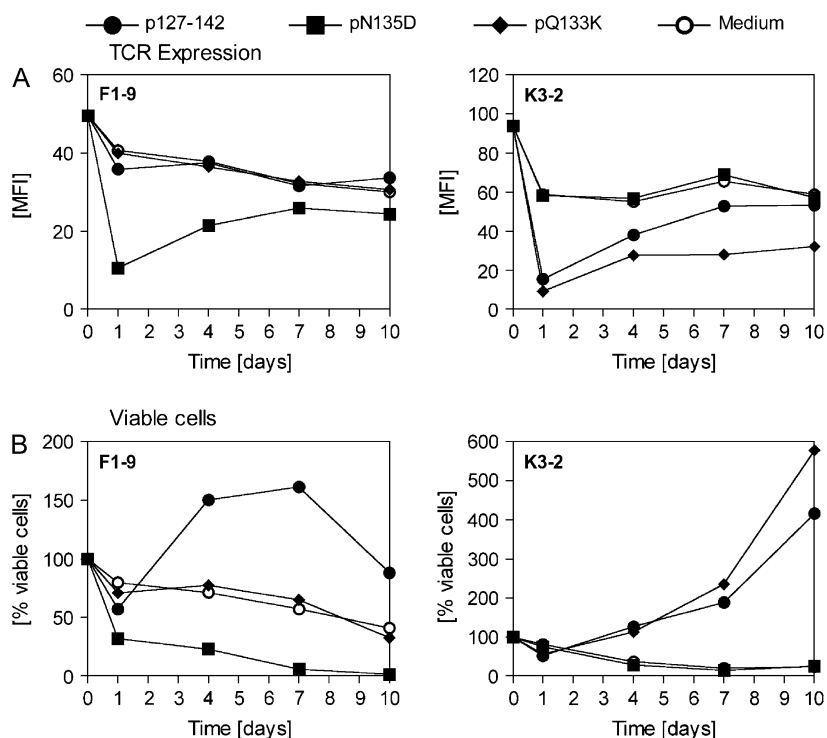


**Fig. 5.** The effect of stimulation with p127-142 or its heteroclitic analogs on Bos d 2-specific T-cell clones F1-9 and K3-2. The TCR expression (A) and the number of viable T cells (percentage of the viable cells on day 0) (B) were monitored at different time points after stimulation with the peptides (5 μM). The results are representative of three independent experiments.

decreased slowly over time but was still observable on day 10 (Fig. 5A).

Upon stimulation with p127–142, both F1-9 and K3-2 expanded steadily after an initial decrease on day 1 (Fig. 5B). In contrast, the number of viable T cells in the non-stimulated cultures slowly declined during the culture period. Interestingly, when clone F1-9 was stimulated with the heteroclitic analog pN135D, the cells rapidly started to die. The extent of cell death on day 10 varied between 88% and 98% in independent experiments. The heteroclitic analog pQ133K, however, was not able to induce the death of clone K3-2. The clone expanded throughout the culture period in numbers comparable with those observed with p127–142 (Fig. 5B).

To analyze whether the long-lasting TCR down-regulation of K3-2 observed upon stimulation with pQ133K had an impact on the capacity of the clone to respond to stimulation with the natural peptide ligand, live K3-2 cells were separated 10 days after stimulation with either p127–142 or pQ133K and re-stimulated with p127–142 and fresh APCs (Fig. 6). The T cells initially stimulated with p127–142 proliferated as vigorously as the unstimulated T cells. In contrast, the T cells initially stimulated with pQ133K showed a diminished proliferative response upon re-stimulation with p127–142 on day 10, indicating that a hyporesponsive state was induced upon stimulation with the heteroclitic peptide analog.

## Discussion

Recent trials of allergen immunotherapy employing short allergen-derived peptides have shown promising results (4–7). The suggested mechanisms of T-cell tolerance in peptide-based allergen immunotherapy include deviation from a  $T_H2$ - to  $T_H1$ -type cytokine profile, activation-induced cell death, anergy and the induction of regulatory T cells (3, 30).

One way to improve the efficacy of peptide immunotherapy is to use heteroclitic analogs of the immunodominant peptides (31). The heteroclitic analogs of a natural epitope have been described for both CD4+ (20, 32, 33) and CD8+ T cells (34, 35) and their immunotherapeutic potential has been demonstrated in murine models of cancer (13), diabetes (14) and allergic asthma (18).

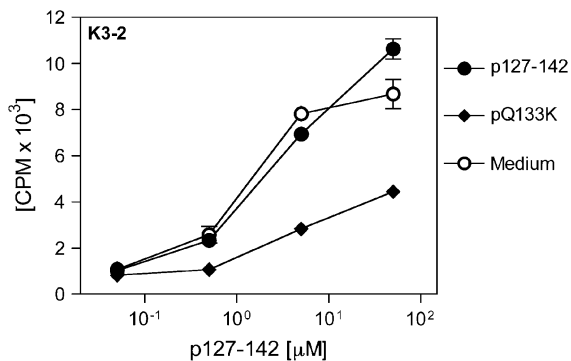


**Fig. 6.** Proliferative response of clone K3-2 upon re-stimulation at different concentrations of p127–142 on day 10 after the initial stimulation on day 0 with either p127–142 or pQ133K (5  $\mu$ M). Cells that were not stimulated on day 0 served as control (medium). The results are representative of three independent experiments.

In this study, we have demonstrated that the heteroclitic analogs of the immunodominant epitope of lipocalin allergen Bos d 2 were able to induce both TCR down-regulation and CD25 up-regulation on specific human T-cell clones at lower peptide concentrations than the natural ligand (Fig. 1). We are now able to show directly by MHC class II tetramer staining that the phenomena result from the increased TCR affinity of the clones for the heteroclitic analogs (Fig. 2). These findings are in accordance with our hypothesis that the immunodominant epitope of Bos d 2 is a sub-optimal ligand for human T cells (20).

Strong T-cell stimulation, either by high antigen dose (36–38) or high affinity of the TCR–MHC interaction (37, 39, 40), has been shown to favor the induction of  $T_H1$ -type responses. In this study, we were able to show that a strong stimulus through TCR modified the balance of cytokine production in favor of IFN- $\gamma$  in the established Bos d 2-specific T-cell clones (Fig. 4). Consequently, the clones F1-9 and K3-2 elicited higher  $T_H1/T_H2$  cytokine ratios upon stimulation with the heteroclitic analogs than with the natural peptide ligand at equimolar concentrations. Increasing IFN- $\gamma$ /IL-4 ratios with the increasing strength of stimulation has also been reported with other allergen-specific T-cell clones (41, 42). Interestingly, the increased  $T_H1$  to  $T_H2$  cytokine production by allergen-specific T cells has also been observed upon successful SIT (43–45), which is associated with the administration of considerably higher doses of allergen than those encountered naturally (2).

Previous investigations indicate that the supraoptimal stimulation of T cells with high doses of antigen leads to T-cell tolerance by induction of hyporesponsiveness and cell death (46–50). We observed both phenomena during the 10-day follow-up after *in vitro* stimulation of clones F1-9 and K3-2 with the heteroclitic analogs. Clone F1-9 died rapidly after the stimulation with pN135D (Fig. 5B), although it exhibited considerable proliferation (Fig. 1), a phenomenon characteristic of activation-induced cell death (51). Clone K3-2, on the other hand, became hyporesponsive to p127–142 after stimulation with pQ133K (Fig. 6). In contrast, stimulation of the clones with the natural peptide appeared to favor the expansion of the cells (Fig. 5B). It seems that the heteroclitic analogs efficiently provided the critical TCR occupancy necessary for the induction of tolerance (52, 53).

Recombinant MHC class II tetramers have been successfully used in detecting CD4+ T cells specific for viral, bacterial and autoantigens (21, 54–58). To our knowledge, this study is the first one to report specific staining of allergen-specific CD4+ T cells by MHC class II tetramers. Two of the three DRB1\*0401-restricted T-cell clones were stained with the DRB1\*0401/p127–142 tetramer, whereas clone F1-9 failed to stain with it. This clone appears to have a lower TCR affinity for p127–142, since the TCR down-regulation observed with the clone upon stimulation with the natural peptide was also weaker than with the other T-cell clones. Despite the lower TCR affinity and inability to stain with the tetramer, effector functions could still be induced upon stimulation with p127–142. It is of interest to note that autoreactive T cells with a low TCR affinity for an autoantigen also seemed to have a reduced capability to bind MHC class II tetramers (56, 58).

In summary, our results suggest that the heteroclitic analogs of the immunodominant epitope of Bos d 2 can have favorable

immunomodulatory effects on allergen-specific T cells. The heteroclitic analogs proved to be superior to the natural peptide ligand both in deviating the response towards T<sub>H</sub>1 and in inducing cell death or hyporesponsiveness of specific T-cell clones *in vitro*. Further analysis of the T-cell response of Bos d 2-allergic patients against the heteroclitic analogs will provide insight on the possible clinical significance of our findings.

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## Abbreviations

|       |                               |
|-------|-------------------------------|
| 7-AAD | 7-amino-actinomycin D         |
| APC   | antigen-presenting cell       |
| APL   | altered peptide ligand        |
| MFI   | median fluorescence intensity |
| PSA   | prostate-specific antigen     |
| SIT   | specific immunotherapy        |

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