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Enhancement of the inhibitory effect of an IL-15 antagonist peptide by alanine scanning

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IL-15 is a proinflammatory cytokine that acts early in the inflammatory response and has been associated with several autoimmune diseases including rheumatoid arthritis, where it had been proposed as a therapeutic target. We recently reported an IL-15 antagonist peptide corresponding to sequence 36–45 of IL-15 (KVTAMKCFLL) named P8, which specifically binds to IL-15Rα and inhibits IL-15 biological activity with a half maximal inhibitory concentration (IC50) of 130 μM in CTLL-2 proliferation assay. In order to improve binding of peptide P8 to the receptor IL-15Rα, we used an Ala scan strategy to study contribution of each individual amino acid to the peptide's antagonist effect. Here, we found that Phe and Cys are important for peptide binding to IL-15Rα. We also investigated other single site mutations and replaced the second Lys in the sequence by the polar non-charged amino acid threonine. The resulting peptide [K6T]P8 exhibited a higher activity than P8 with an IC50 of 24 μM. We also found that this peptide was more active than peptide P8 in the inhibition of TNFα secretion by synovial cells from rheumatoid arthritis patients. The peptide [K6T]P8 described in this work is a new type of IL-15 antagonist and constitutes a potential therapeutic agent for rheumatoid arthritis. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antagonist; cytokine; IL-15; peptide; receptor alpha

Introduction

IL-15 is a member of the four α-helix bundle cytokine family. Pleiotropic expression of IL-15 and its receptor suggests a multiplicity of functions for IL-15 in multiple cell types and tissues [1]. Effects of IL-15 are mediated by a trimeric membrane receptor comprised of β and γ subunits of the IL-2 receptor but a different α subunit [2]. IL-15Rα alone, which can also be found in a soluble form, displays a high binding affinity for IL-15, and its mRNA is widely distributed in the tissue [3]. In fact, IL-15Rα plays an important role in different mechanisms of action described for IL-15, and the IL-15/IL-15Rα complex is more active than IL-15 alone [4,5].

Current available data support a proinflammatory role for IL-15 in a number of immunopathologies, such as rheumatoid arthritis, inflammatory bowel disease, sarcoidosis, and multiple sclerosis [6–9]. Several studies have generated different IL-15 antagonists, such as neutralizing antibodies directed against IL-15 itself or alternatively, against IL-2R/IL-15Rβ, mutant IL-15 molecules, and soluble fragments of the IL-15Rα chain linked to the immunoglobulin Fc element, that have been shown to be effective both in animal models and humans [10–12].

In a previous study, we have identified a small peptide corresponding to the sequence 36–45 if IL-15 (KVTAMKCFLL) named P8, which specifically binds to IL-15Rα and exhibits an antagonist effect on IL-15 activity [13]. Here we prepared a series of single point, Ala-substituted P8 peptide analogs to evaluate contribution of their individual amino acid side chains to IL-15Rα binding. As result, we have identified the peptide [K6T]P8 exhibiting a tenfold enhanced antagonist activity. Moreover, we have demonstrated that both wild type P8 and this more active analog [K6T]P8 inhibit secretion of TNFα, a validated target in RA [14]. Therefore, we propose a new type of IL-15 antagonist that could be used for treatment of diseases involving signaling through IL-15/IL-15Rα interaction.

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Abbreviations: *AAA amino acid analysis; DIC **1**,**3**-diisopropylcarbodiimide; DMF N,N-dimethylformamide; DIEA diisopropylethylamine; DMSO dimethyl sulfoxide; EDT-1 **2**-ethanedithiol; Fmoc/tBu **9**-fluorenylmethyloxycarbonyl/tert-butyl; Fmoc-AM (p-[(R,S)-α-[1-(9H-fluoren-9-yl)methoxy-formamido]-**2**,**4**-dimethyl-benzyl] phenoxyacetic acid; HOBt **1**-hydroxybenzotriazole; RP reverse phase; HPLC high-performance liquid chromatography; MBHA **4**-methylbenzhydrylamine; ESI-MS electro spray ionization mass spectrometry; PBS phosphate-buffered saline; TFA trifluoroacetic acid; TIS triisopropylsilane; RA rheumatoid arthritis; TNFα tumor necrosis factor; IL interleukin, IL-15R interleukin-15 receptor; ELISA enzyme-linked immunosorbent assay.*

Results and Discussion

Because of the high-affinity interaction between IL-15 and IL-15Rα, it was important to optimize binding of the P8 peptide to IL-15Rα in order to reduce its 50% inhibitory dose (IC50 = 130 μM). To study the contribution of each amino acid to its antagonist effect, we synthesized a family of single Ala mutants of this peptide. Ala is often selected for such scans because it is assumed that single Ala substitutions do not disrupt the secondary structure of the peptide. All peptides were obtained with more than 96% of purity, and their molecular mass was verified by mass spectrometry (Table 1). Peptides were then evaluated by preincubation with IL-15Rα, and subsequently, the resulting mixtures were added onto IL-15-coated surfaces in order to measure the ability of each synthetic peptide to competitively inhibit IL-15/IL-15Rα complex formation. In previous studies, we had observed that mutant [C7A]P8 was not able to inhibit formation of the IL-15/IL-15Rα complex in the competitive ELISA [13], whereas here we found that [F8A]P8 mutant showed only 30% inhibition of this complex formation. Moreover, mutations of Leu residues in the C-terminus of P8, i.e. [L9A] P8 and [L10A] P8 peptides, affect their inhibitory activity although to a lesser extent as they showed 40% and 50% of inhibition, respectively. On the other hand, substitutions of the Lys residues by Ala on P8, led to a significant decrease in solubility of the peptides [K1A]P8 and [K6A]P8, making it difficult to evaluate these mutants. Other mutants such as [V2A]P8, [T3A]P8, and [M5A]P8 showed inhibition potencies similar to wild type P8 (Figure 1). These mutations did not affect the capacity of P8 to inhibit formation of the IL-15/IL-15Rα complex.

In order to evaluate the effect of the different substitutions by a method based on IL-15 biological activity, the antagonist effect of the analogs was evaluated on a CTLL-2 cells proliferation assay, a murine cell line that expresses the trimeric receptor IL-15Rα$\beta\gamma$. For this purpose, we chose an IL-15 dose of 300 pg/ml, corresponding to the beginning of the proliferation curve's plateau, in order to assess high-potency antagonists. In agreement with aforementioned results, we found that substitutions in P8, which affect binding of IL-15 to IL-15Rα, showed a reduced antagonist activity. Correspondingly as previously reported with

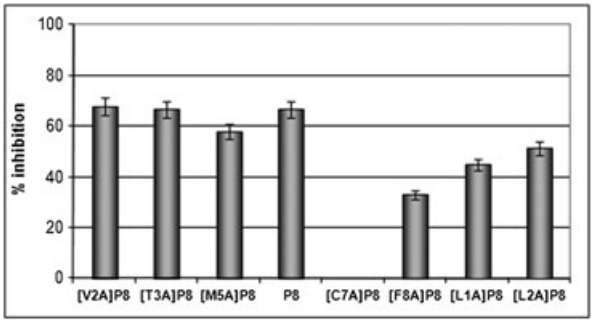

Figure 1. Graphic represents capacity of analogous peptides to displace binding of IL-15Rα to IL-15. Results are expressed as percentage of inhibition of the IL-15/IL-15Rα complex formation and represent mean values ± standard deviation from three independent experiments.

mutant [C7A]P8, substitution of Phe, [F8A]P8, also abrogates P8 inhibitory activity and its capacity as an IL-15 antagonist (Figure 2A, Table 1). As for Leu residues at the C-terminus of P8, their substitution by Ala resulted in either case to a twofold reduction of its antagonist effect (Figure 2B and Table 1). Both Leu[44] and Leu[45] were proposed as part of an IL-15Rα binding sequence identified by Bernard *et al.* [15] and could well contribute to the interaction between peptide P8 and IL-15Rα.

Others substitutions such as [V2A]P8, [M5A]P8, and [T3A]P8 did not affect the antagonist potency (Figure 2C and Table 1) nor binding to IL-15Rα.

Peptide P8 contains two Lys residues in positions 36 and 41 of the IL-15 molecule. The substitution of each one by Ala, [K1A]P8 and [K6A]P8, was found to affect the solubility and making it difficult to evaluate these variants. Therefore, Lys[41] on P8 was replaced with other aminoacids, such as Glu with its charged polar side chain and Thr with the polar but uncharged side chain. Both substitutions improved the peptide solubility but interestingly, we found that replacement of this Lys[41] by Glu, [K6E]P8, completely abolished the antagonist activity. Conversely, replacement of Lys[41] by Thr, generated the peptide [K6T]P8 with higher antagonist activity than P8 in CTLL-2 cells proliferation assays, showing an IC50 value of 24 μM (Figure 3, Table 1). The inhibitory

Table 1. Analytical data of the synthetic peptides and the concentrations of mutants giving half-maximal inhibitory effects (IC50 values) in CTLL-2 cell line. ND, not determined

Sequence	RP-HPLC retention time (min)	Purity (%)	Monoisotopic mass (Da)		IC50
			Calculated	Found	μM
1. KVTAMKCFLL P8	21.12	98.69	1151.65	1151.64	130
2. AVTAMKCFLL [K1A]P8	22.02	98.60	1094.60	1094.62	ND
3. KATAMKCFLL [V2A]P8	20.60	97.90	1123.62	1123.64	130
4. KVAAMKCFLL [T3A]P8	21.08	97.77	1121.64	1121.66	130
5. KVTAAKCFLL [M5A]P8	19.53	98.41	1091.65	1091.67	130
6. KVTAMACFLL [K6A]P8	23.95	96.67	1094.60	1094.60	ND
7. KVTAMKAFLL [C7A]P8	20.50	98.63	1119.68	1119.68	inactive
8. KVTAMKCALL [F8A]P8	18.27	98.01	1075.62	1075.62	inactive
9. KVTAMKCFAL [L9A]P8	18.81	96.66	1109.61	1109.61	200
10. KVTAMKCFLA [L10A]P8	18.69	97.09	1109.61	1109.60	260
11. KVTAMKSFLL [C7S]P8	19.16	99.18	1135.68	1135.65	inactive
12. KVTAMTCFLL [K6T]P8	22.71	97.98	1124.61	1124.60	24.6
13. KVTAMECFLL [K6E]P8	23.78	96.37	1152.60	1152.61	inactive
14. KVTAMTCFLL dimer [K6T]P8 dimer	25.74	98.29	2247.20	2247.18	8.0

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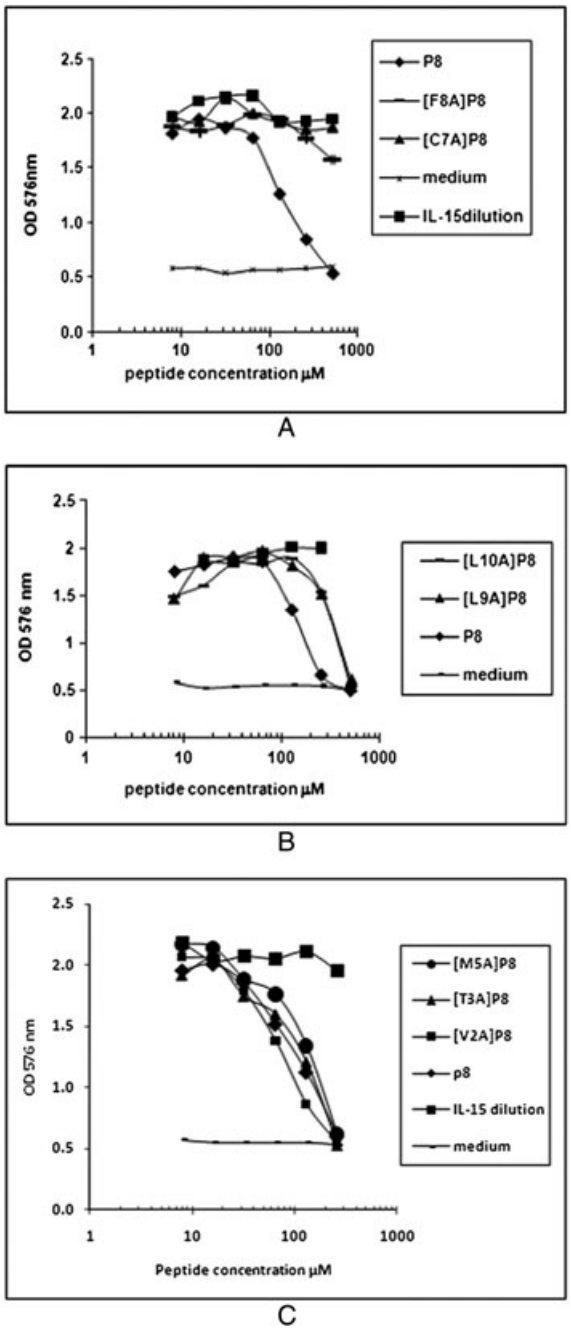

Figure 2. Effect of P8 and P8 mutant peptides on IL-15 proliferative activity in CTLL-2 cells. CTLL-2 were cultured for 72 h in presence of 300 pg/ml of IL-15 (■) or 300 pg/ml of IL-15 plus increasing concentrations of P8(♦), medium(-) or mutants (A) [F8A]P8(—), [C7A]P8(▲), (B) [L9A]P8 (▲), [L10A]P8(—),and (C) [M5A]P8(•), [T3A]P8 (▲), [V2A]P8(■). Cell proliferation was evaluated by MTT staining.

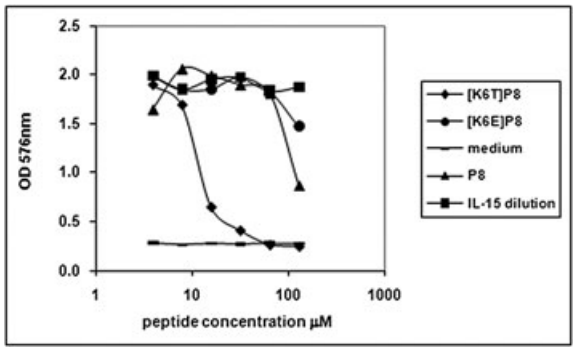

Figure 3. Effect of P8 Lys mutants on IL-15 proliferative activity in CTLL-2 cells. The cells were cultured for 72 h in presence of 300 pg/ml of IL-15 (■) or 300 pg/ml of IL-15 plus increasing concentrations of P8, mutants or medium (—), [K6E]P8 (●), [K6T]P8 (♦), P8 (▲). Cell proliferation was evaluated by MTT staining.

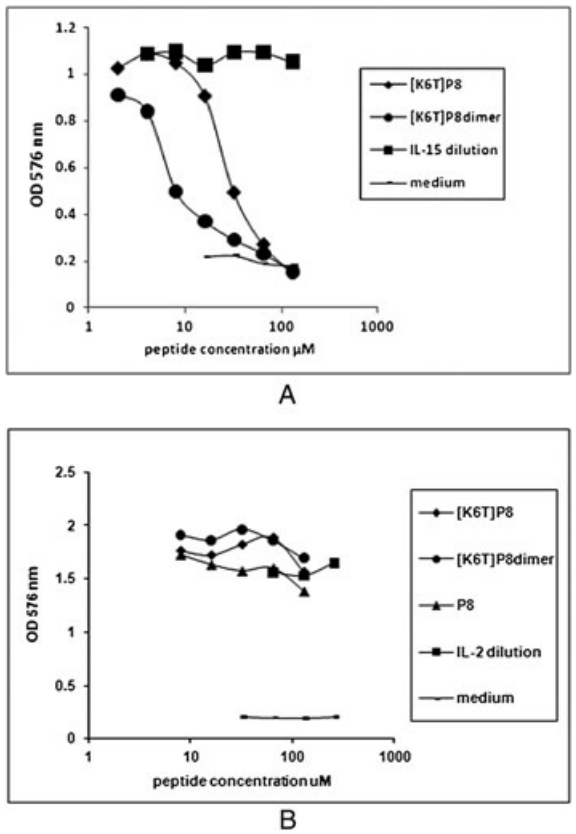

Figure 4. Effect of P8 and [K6T]P8 peptides on IL-15 and IL-2–dependent proliferative activity in CTLL-2 cells. Cells were cultured for 72 h in presence of 10 000 IU/ml of (A) IL-2 (■) or (B) IL-15 300 pg/ml (■), plus increasing concentrations of P8 (▲), [K6T]P8 (♦) or medium (—), [K6T]P8 dimer (•). Cell proliferation was evaluated by MTT staining.

effect of the [K6T]P8 peptide was dose-dependent. Additionally, [K6T]P8 was unable to affect IL-2-induced proliferation of these cells, demonstrating specificity towards inhibition of the IL-15/IL-15Rα interaction (Figure 4A and B). These results suggested that the positive charge of the side chain in this position is important for peptide's antagonist effect, on the basis of the fact that creating a negative charge completely abrogates P8 inhibitory activity.

Previously, we have reported that P8 dimer was more active than the monomeric form [13]. To evaluated dimer activity, [K6T] P8 was obtained in a completely dimeric form by oxidation of the thiol group with 20% DMSO. CTLL-2 proliferation assays showed that the dimer had a higher inhibitory activity (IC50 = 8 µM) than the monomeric form (Figure 4B, Table 1). This finding confirms that even this peptide is more active in its dimeric form as previously reported for P8 [13].

The results of these studies suggest that both Cys and Phe residues are important for binding to IL-15Rα and therefore, for peptide inhibitory activity. The corresponding Cys^{42} residue in the parent IL-15 protein is involved in a disulfide bridge with Cys^{88}.

Olsen *et al*. studied the crystal structure of the IL-15/IL-15Rα complex and stated that even when most of the interactions at the IL-15/IL-15Rα interface are mediated by hydrogen bonds, there is a prominent hydrophobic pocket where the disulfide bridge between Cys^{42} and Cys^{88} is located. Additionally, this group found that helix B and the loop between helix A and helix B on IL-15 account for a major portion of the ligand–receptor interface in the IL-15/IL-15Rα complex, and all amino acid residues of P8 are situated in helix B [16,17]

Our results indicate that P8 and its analogs interact with IL-15Rα mainly through hydrophobic interactions with Cys, Phe and two Leu amino acids contributing to them. In agreement with this, when the Lys closer to Cys is replaced with Glu, P8 inhibitory activity is completely abrogated, whereas substitution with a non-charged amino acid such as Thr generates a peptide with an enhanced inhibitory activity.

In order to assess activity of P8 and its analog [K6T]P8 on other IL-15-induced biological effects, we measured the effect of these peptides on TNFα secretion. It had been proposed that an IL-15 antagonist could be useful in treatment of some autoimmune diseases in which IL-15 acts as proinflammatory cytokine. Particularly, high levels of IL-15 have been found in synovial fluids from RA patients [18], and it is known that in response to IL-15, synovial T cells secrete TNFα directly and induce TNFα synthesis by macrophages through cognate interactions [19,20].

Synovial cells from RA patients with high levels of IL-15 in synovial fluids were incubated with P8 and the mutant [K6T]P8 in presence of IL-15 for 72 h, and then TNFα levels were determined by ELISA. We found that both peptides inhibited TNFα secretion from a pool of synovial cells and, in agreement with previous results obtained for IL-15-dependent cell lines, [K6T]P8 was more active than P8 peptide in the inhibition of TNFα secretion (Figure 5A). We also evaluated [K6T]P8 dimer, and its effect was dose-dependent in these experiments (Figure 5B). These results support that this peptide could be useful for treatment of RA taking in consideration that TNFα is an important and validated target in RA.

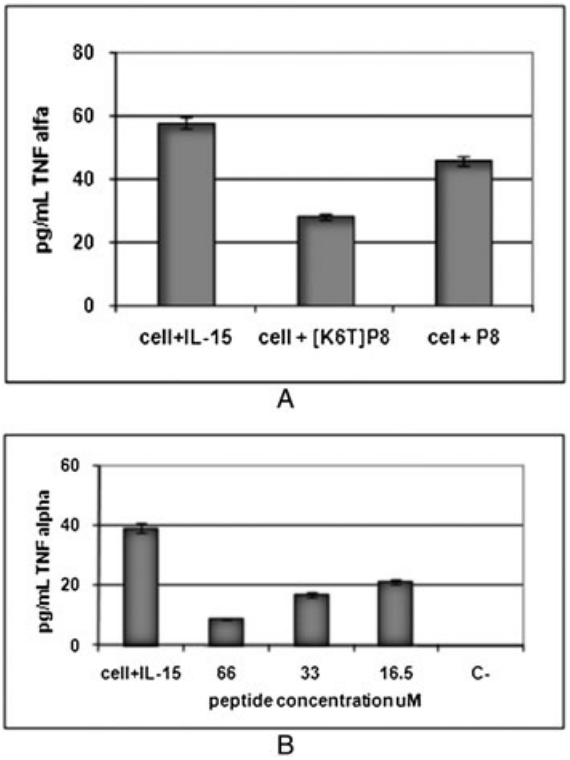

Figure 5. Inhibition of IL-15-mediated production of TNFα in synovial fluid cells from RA patients. (A) Effects of [K6T]P8 and P8 on TNFα secretion are compared. Synovial fluid cells were incubated in 96-well plates at 2×10^5 cells per well with IL-15 plus 20 μg/ml of P8 and [k6T]P8 peptides and (B) different doses of dimer [K6T]P8 peptide. After incubation for 48 h, supernatants were collected and stored at −70 °C until evaluation. Levels of TNFα were quantified by ELISA

Materials and Methods

Peptide Synthesis

The peptides were synthesized on solid phase using the Fmoc/tBu chemistry on Fmoc-AM-MBHA resin. Removal of the Fmoc group was carried out with 20% of piperidine in DMF, and the Fmoc-amino acids were coupled with DIC/HOBt activation. Cleavage from the resin and removal of side chain-protecting groups were accomplished by treatment with TFA/H_2O/EDT/TIS (95/2.5/2.5/1) for 2 h, and the peptides were then precipitated with cold ether, dissolved in 40% acetonitrile/water, and lyophilized [21]. Peptides were purified by RP-HPLC and identified by mass spectrometry (Table 1).

Peptide Dimerization

Peptide [K6T]P8 was dimerized by oxidation of the Cys residue with 20% DMSO in water [22]. Briefly, the peptide was dissolved in water at 4 mg/ml, pH was adjusted to 6 with 25% ammonium hydroxide, and then DMSO was added to a final concentration of 20%. Completion of the oxidation reaction was monitored by RP-HPLC and ESI-MS. Finally, the dimer was purified by RP-HPLC.

Chromatography

Peptides were analyzed on an AKTA 100 (GE Healthcare USA, Piscataway, NJ, USA) HPLC system. Separation was achieved in an RP-C18 column (4.6 × 150 mm^2, 5 μm) (Vydac, Grace, Deerfield, IL, USA). A linear gradient of 5–60% of B for 35 min and a flow rate of 0.8 ml/min were used. Solvent A: 0.1% of TFA in water. Solvent B: 0.05% of TFA in acetonitrile. Chromatograms were acquired at 226 nm, using UNICORN 4.11 (GE Healthcare USA) software package for data processing of the RP-HPLC chromatograms. Purification of peptides was performed on a LaChrom (Merck Hitachi, Darmstadt, Germany) HPLC system, employing an RP-C18 column (Vydac, 25 × 250 mm^2, 25 μm). A linear gradient of 15–45% of B for 50 min and a flow rate of 5 ml/min were used. Absorbance was monitored at 226 nm.

Mass Spectrometry

ESI-MS was performed in a hybrid quadrupole-time-of-flight (Q-TOF2) instrument (Waters, Milford, MA, USA) fitted with a nanospray ion source. Capillary and cone voltage were set to 900 and 35 V, respectively. Data acquisition and processing were performed with the MassLynx (version 3.5) package (Waters).

ELISA for Measurement of Binding to Peptide/IL-15Rα

ELISA plates were coated with 1 μg/ml of IL-15/well in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with

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PBS containing 1% BSA, 50 µl of peptide (20 µg/ml) and 50 µl of IL-15Rα-Fc at 0.125 µg/ml were added and incubated at 37 °C for 1 h, followed by washing with PBS containing 0.1% Tween 20. Bound IL-15Rα-Fc complexes were detected with HRP-conjugated goat anti-human IgG (Sigma) at 37 °C for 1 h, followed by washing. Reaction was visualized by addition of the substrate solution [**3**,**3**′,**5**,**5**′-tetramethyl-benzidine (TMB)] and absorbance at 450 nm was measured by an ELISA plate reader (Biotrak, GE Healthcare USA). Results are reported as a mean of triplicate experiments.

The inhibition of complex formation (%) was calculated using the following formula:

$$\{1 - (([OD]\text{sample} - [O.D]\ \text{min})/([OD]\ \text{max} - [O.D]\ \text{min}))\} \times 100$$

[OD]sample, optical density in presence of analogous peptide; [OD]min, background of the assay; [O.D]max, optical density in presence of IL-15Rα.

Effect of Peptides on Proliferation of CTLL2 Cell Line

For evaluation of the antagonist effects of the peptides, serial dilutions of the peptide under examination were performed in 96-well plates (Costar, Corning Inc., Corning, NY USA) in a volume of 25 µl of RPMI medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco). Then, previously washed CTLL-2 cells were added in amounts of 5×10^3 cells/well and further incubated for 30 min at 5% CO_2 and 37 °C. Afterwards, 300 pg of IL-15 was added to each well, and the plate was incubated for 72 h in the same conditions. Proliferation was measured by MTT mitochondrial staining. Results are reported as a mean of triplicate experiments.

Inhibition of IL-15-Mediated TNFα Production in Synovial Fluid Cells of RA Patients

After obtaining a written informed consent, synovial fluid from RA patients was extracted and incubated with hyaluronidase (10 µg/ml of fluid) for 45 min at 37 °C. Synovial fluid cells were obtained after centrifugation at 1200 rpm for 10 min.

Cells were incubated in 96-well plates at 2×10^5 cells per well either with 50 µg/ml of peptide, or 60 ng/ml of IL-15, or a combination of both. After 48-h incubation, supernatants were collected and stored at −70 °C until further evaluation. TNFα concentration was determined by ELISA (DTA50, R&D Systems, Minneapolis, MN, USA).

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