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Biological and Chemical Research, Volume 2015, 329-337 | Science Signpost Publishing

CNI-1493 Administration Improves the Efficacy of Cytotoxic T Lymphocytes

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Received: November 17, 2014 / Accepted: September 21, 2015 / Published: November 25, 2015

Abstract: CNI-1493, is a synthetic guanylhydrazone with anti-inflammatory properties. The most important application of CNI-1493 is the treatment of Crohn's disease. Adenovirus expressing ovalbumin (AdOVA) induces an increase, as a short peak, of IL-12 shortly after administration, and increased ovalbumin-specific cytotoxic T lymphocyte cells.

Aim of study: To evaluate the safety and immunological effects of the CNI-1493 on the in vivo activity of cytotoxic T lymphocyte cells (CTLs), by using AdOVA immunized C57Bl/6 mice.

Materials and methods: AdOVA were given i.v. at the same time point to two groups of naïve C57Bl/6 WT mice; Gp1: Received CNI-1493, AdOVA and target cells and Gp2: Received AdOVA and target cells. In addition to the above two groups, a control group (Gp3): Received target cells (Neither CNI-1493 nor AdOVA). Five hours, after the target cells administration, the spleens were taken out of the experimental groups and homogenised in 1xPBS. The ratio of lysed target cells was determined by flow cytometry

Results: The OVA specific lysis of target cells in immunized C57Bl/6 mice of the experimental groups were 47.2% in G1 and 81.3% in G2.

Conclusion: CNI-1493 is compound which is safe and undamaging to the immunological function of the cytotoxic T lymphocyte cells.

Keywords: CNI-1493, ovalbumin, specific cytotoxic T lymphocyte cells, and immunized C57Bl/6 mice

1. Introduction

CNI-1493, (*N*,*N*′-bis[3,5–bis[1(aminoimino-methyl)hydrazonoethyl]phenyl] decanedi-amide tetrahydrochlo-ride), formerly known as semapimod, is a synthetic guanylhydrazone with anti-inflammatory properties [5], including TNF and NO [6]. Initial studies suggested that the mechanism of the

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anti-inflammatory action of CNI-1493 involves the inhibition of the phosphorylation of p38 MAPK, which plays an integral role in the translation of mRNA of pro-inflammatory cytokines such as TNF [5, 23, 24]. In addition it involves the inhibition of JNK signaling pathways [9]. Thus, the anti-inflammatory properties of CNI-1493 have been applied in a variety of diseases. Suppression of renal cancer and melanoma was shown in a clinical phase I trial study [1]. CNI-1493 administration reduced myocardial inflammation and myocyte apoptosis. Recently, the compound was also shown to inhibit trans activation of human immunodeficiency virus type 1 (HIV-1) protein Rev [14], which is essential for virus replication; inhibition was also observed in multi-drug-resistant HIV strains. This inhibitory effect was based on suppression of deoxyhypusine synthase (DHS) and subsequently of eIF-5A biosynthesis, which is an essential cellular cofactor of the HIV-1 Rev regulatory protein [4]. The most important application of CNI-1493 [17], which is now under evaluation in clinical trail phase II, is the treatment of Crohn's disease, a chronic inflammatory disease of the gut. Moreover, treatment with CNI-1493 resulted in a profound inhibition of lipopoly-saccharide (LPS)- induced production of TNF, IL-1, IL-6, and IL-8 [7]. In addition to that, our research group had proven that CNI-1493 inhibits *P. falciparum* DHS and it provide in vivo protection against cerebral malaria of mice infected with *P. berghei* [15, 21].

Interleukin-12 (IL-12) is a heterodimeric cytokine that is produced predominantly by macrophages, dendritic cells (DCs), and a variety of other immune cells and serves as a key regulator of cell-mediated immunity [2]. IL-12 has potent biological effects in vitro and in vivo. In addition, it has been shown to (a) induce IFN-γ, production, (b) augment cytotoxic function and proliferation of NK and activated T cells, and (c) promote Th-1 type cytokine responses [29]. In addition, Endogenous IL-12 is clearly important for protective immune responses during acute infections with intracellular parasites [22] and bacteria [26].

In contrast to that, Orange et al, 1995 [20], suggested a pathway by which infection with agents inducing IL-12 and/or TNF, during a viral infection, might dramatically limit protective anti-viral immune responses and induce physiologically adverse effects on the host. In agreement with the above findings, when given systemically in a phase I clinical trial, recombinant IL-12 induced multiple serious adverse effects, including renal and systemic toxicity [10, 16]. High-dose levels were linked to temporary immune suppression, which would be unfavorable for effective immunotherapy. However, low doses of IL-12 (1-10 ng/d) enhance, whereas high doses (100-1,000 ng/d) inhibit $CD8^+$ T cell responses. In fact, both $CD8^+$ T cell expansion and presence of virus-specific CTLs are reduced with the higher doses of IL-12 [12]. As high dose IL-12 administration induces numerous necrotic lesions and apoptotic cells in lymphoid tissue, the effects on T cell responses appear to be associated with cell death.

Adenovirus expressing ovalbumin (AdOVA) induce an increase, as a short peak, in IL-12 level [13, 18], shortly after administration, and increased ovalbumin (OVA)-specific cytotoxic T lymphocyte cells (CTL) [28].

Aim of study: To evaluate, how does the administration of CNI-1493 will affect the in vivo cytotoxicity of CTLs?.

2. Materials and Methods

Animals: The animal studies were performed according to the guidelines of the German animal rights (license number: 50.203.2-BN). Where C57BL/6-WT mice were obtained from Harlan.

CNI-1493 and Adenovirus administration: CNI-1493 was dissolved in DMSO and prepared in PBSx1 at 4mg/kg to be injected intravenously (i.v.).

$1x10^8$ pfu/mouse of AdOVA were administrated i.v. at the same time to two groups of Naïve C57Bl/6WT mice; Gp1 received CNI-1493 (pre-treated group, treated on -5, -3 and -1 day before AdOVA administration), AdOVA and target cells. Gp2 received AdOVA and target cells (Non-CNI-1493 treated). In addition to the above two groups, a control group (Gp3) received target cells (Neither CNI-1493 nor AdOVA). (Figure 1: Illustrates the experimental time points).

Recombinant Adenovirus type 5 expressing OVA were kindly provided by the Institute for Molecular Medicine and Experimental Immunology (IMMEI), Bonn, Germany.

Fig. 1. The experimental time points: days -5, -3, and -1 are the time points of CNI-1493 administration, day 0 is the time point of AdOVA administration, day 5 is the time point of Target cells administration, and splenocytes were harvested 4h after target cells administration.

Target cells administration: Each mouse has received $1x10^7$ cells from the 58L target cells which given i.v. five days after immunization with AdOVA to the all three experimental groups. Five hours later the spleen was taken out and homogenised in 1xPBS. The ratio of lysed target cells was determined by flow cytometry (By using: FACS CANTO Becton Dickinson GmbH, Germany).

The ratio of lysed target cells was determined by the following equation:

$$\% \text{ of spicific lysis} = 100 - \left[100x \frac{\left[\frac{\text{CFSE high}}{\text{CFSE low}} \right] \text{Immunized mouse}}{\left[\frac{\text{CFSE high}}{\text{CFSE low}} \right] \text{Non-Immunized mouse}} \right]$$

Where as CFSE: CarboxyFluoroscein Succinimidyl Ester

58L target cells, generated from splenocytes from syngenic donors, were kindly provided by the Institute for Molecular Medicine and Experimental Immunology (IMMEI), Bonn, Germany.

The experimental principle: Immunized mice should record reduced numbers of target cells because of activated cytotoxic T lymphocyte cells, whereas naïve animals should have stable numbers of target cells.

58L target cells were labelled with 1 μM CFSE to allow visualization via flow cytometry and labelled with OVA antigen-specific class I peptides, these target cells are recognized by CTLs and are lysed by antigen-specific CTLs of immunized animals. Internalization of adenovirus (Ad) into target cells is mediated by two steps: The fiber knob of Ad particles initially attaches to coxsackie-adenovirus receptor (CAR) on the cell surface [3], and then to $\alpha_v\beta_3$- or $\alpha_v\beta_5$-integrins which interact with the Arg-Gly-Asp motif in the Ad-penton base and hence facilitate internalization of the virion [27].

3. Results

In particular, the release of increased levels of pro-inflammatory cytokines (i.e. IL-12, IFN-γ and TNF) is of importance in different stages of systemic, infectious diseases, because it has a beneficial role in host defense. However, the pro-inflammatory cytokines could be harmful to the host if produced excessively. The results described here document the effects of CNI-1493 on the CTLs in vivo cytotoxicity.

CNI-1493 in Vivo cytotoxicity assay: 4 mg/kg of CNI-1493 was administrated i.v., for 5 days before Ad-OVA infection (Gp1). The generation of OVA specific cytotoxic T cells (CTLs) in CNI-1493 treated or untreated mice was evaluated by flow cytometry. The OVA specific lysis of target cells in immunized C57Bl/6 mice of the experimental groups were 47.2% in Gp1 and 81.3% in Gp2 as shown in (Fig. 2). On the other hand, no specific lysis of target cells in Gp3 (specific lysis of target cells was 0%).

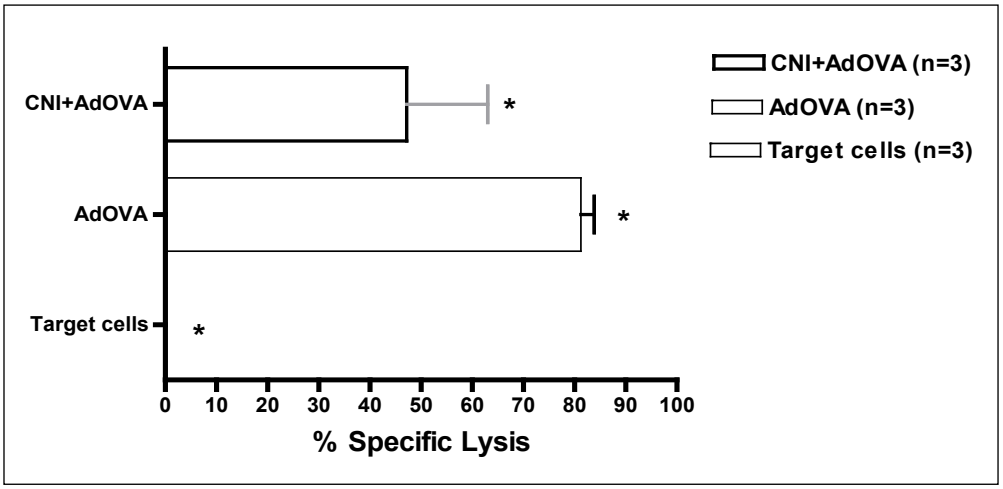

Fig. 2. In vivo cytotoxicity assay of CNI-1493. Mean lysis of target cells by antigen specific T cells of AdOVA infected mice is 81.3 %. CNI-1493 administration non-significantly reduced the cytotoxic activity of antigen specific T cells in the spleen. 4mg/kg of CNI-1493 were given i.v. to C57Bl/6 mice on d -5, -3 and d-1 pre AdOVA administration. Each mouse received $1x10^7$ target cells, which given i.v. on d 5 post AdOVA administration. Splenocytes were harvested 4 h after target cells administration. * Specific lysis: Gp1 vs Gp3, $P < 0.05$, Gp2 vs Gp3, $P < 0.01$ and by Tukey's Multiple Comparison Test. whereas Gp1 vs Gp2 non-significant P- value.

In another meaning, CNI-1493 suppressed about 34.1% of the in vivo cytotoxicity function of the specific CTLs generated in the spleen (the detected target cells are illustrated in percentages in fig.3).

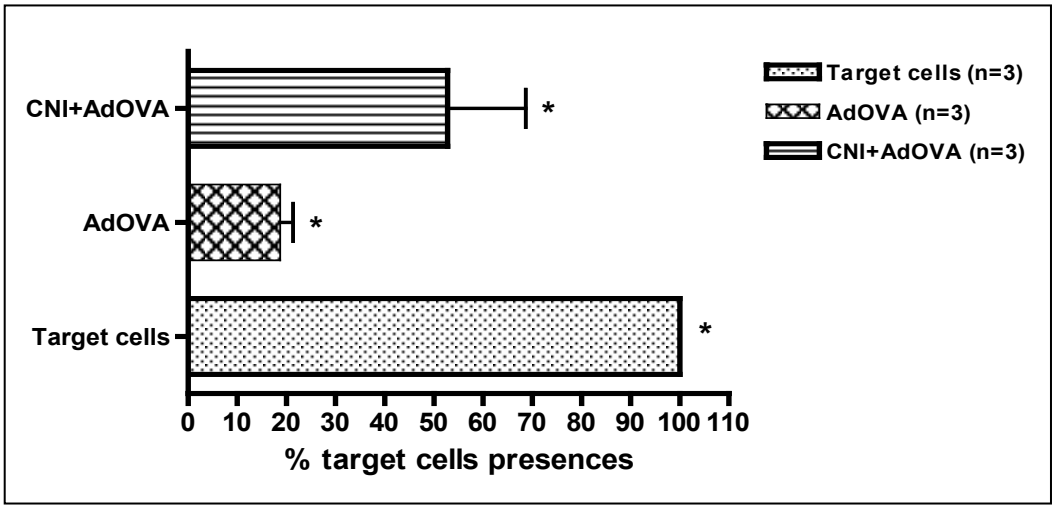

Fig. 3. Percentages of target cells presences in the experimental groups.

* Gp1 vs Gp3 $P < 0.05$, Gp2 vs Gp3 $P < 0.01$, and Gp1 vs Gp2 $P > 0.05$ by Tukey's Multiple Comparison Test.

However, Immunized mice showed significantly reduced numbers of target cells by the activated cytotoxic T lymphocyte cells in the AdOVA immunized C57Bl/6 mice even in the presence of CNI-1493.

4. Discussion

Effective cell-mediated immunity, essential for the protection against chronic intracellular infections and cancer, depends on the concordant activity of $CD8^+$ T cells (CTL) and $CD4^+$ T cells, especially Th1 cells [19]. However, the activation of T cells and NK cells increases the production of the IL-12 receptor (beta1and beta 2), which explains why IL-12 can direct the proliferation and activation of T lymphocytes, NK cells, and NKT cells and can induce both IFN-γ and increased cytotoxic activity [25]. Since, IL-12 enhances the cytolytic activity of cytotoxic T and NK cells, and induces IFN-γ production from T and NK cells [2, 29] therefore, the importance of IL-12 will be considered.

This open-label study was designed to evaluate the safety and immunological effects of the CNI-1493 on the in vivo activity of CTLs, by using AdOVA immunized C57Bl/6 mice.

From the data obtained so far, the CTLs significantly reduced the number of target cells in the presence of CNI-1493 ($P < 0.05$). On the other hand, there were no statistical differences in the cytolytic activity of CTLs in both groups (present and absent of CNI-1493). These outcomes are in agreement with a large body of data in multiple animal models, which suggests that CNI-1493 can be useful to prevent the macrophage mediated sequelae of disease and trauma, including sepsis, cerebral ischemia, and endotoxemia through inhibition of proinflammatory cytokine responses [5, 6, 8, 11, 15, 21, 25].

Hence, this study discovered more safety-related properties of CNI-1493 and improvement in the functioning of CTLs. Additionally, this study provides insights that may be of value to protect from IL-12 toxicity and may prove useful as a research tool to elucidate the distinct mechanisms involved in IL-2 host toxicity, which has not been utilized yet.

However, additional studies on the interaction between CNI-1493 and IL-12 are needed to provide better understanding and to clarify the relationship between them.

5. Conclusion

It can be concluded that CNI-1493 is a compound which is safe and undamaging to the immunological function of the cytotoxic T lymphocyte cells.

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