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IL-2 complex treatment amplifies CD8⁺ T cell mediated immunity following herpes simplex virus-1 infection

Naveen K. Rajasagi¹ and Barry T. Rouse^{1,*}

¹Biomedical & Diagnostic Sciences, College of Veterinary Medicine, The University of Tennessee, Knoxville, TN, 37996-0845

Abstract

CD8⁺ T cells play an important role in controlling numerous virus infections and some tumors and therefore several strategies have been adopted to modulate CD8⁺ T cell responses. One such approach includes treatment with IL-2 bound to a monoclonal antibody against IL-2 (IL-2 complex) which was shown to enhance CD8⁺ T cell responses and provide protection against some cancers and pathogens. This report analyses the value of IL-2 complex therapy to protect against a cutaneous virus infection as occurs with herpes simplex virus-1 (HSV-1) infection. Treatment with IL-2 complex after infection reduced virus levels and lesion severity in a zosteriform model of HSV infection in mice. Furthermore, IL-2 complex treatment expanded HSV-1-gB epitope-specific CD8⁺ T cells, IFN- γ and TNF- α producing CD8⁺ T cells as well as cells that produced more than one cytokine. In addition, IL-2 complex therapy recipients showed enhanced cytolytic activity of CD8⁺ T cells as shown by increased granzyme B expression and lytic granule release. Taken, together, these studies demonstrate that IL-2 complex therapy can be useful to boost protection against a cutaneous virus infection.

Keywords

IL-2 complex; herpes simplex virus-1; CD8 T cells

1. Introduction

Recovery from many viral infections is mediated primarily by CD8⁺ T cells [1]. In such infections whether or not clinically relevant responses occur may depend on the magnitude and function of CD8⁺ T cells. Consequently, in the control of viral infections, finding approaches that expand and activate CD8⁺ T cell responses become an important issue. It is evident from work done mainly in the cancer field that the CD8⁺ T cell responses can be elevated upon therapy with IL-2 complexed with an appropriate clone (clone S4B6) of

*Corresponding author: Dr. Barry T. Rouse, Biomedical & Diagnostic Sciences, M409, Walters Life Sciences Bldg., The University of Tennessee, 1414, Cumberland Ave, Knoxville TN 37996-0845, Phone: 865-974-4026, Fax: 865-974-7817, btr@utk.edu.

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monoclonal antibody against IL-2 [2]. Such complexes increase the activity of IL-2 in vivo, leading to rapid expansion of CD8⁺ T cells and better control of tumors [3]. The approach may also enhance immunity to some systemic bacterial and viral pathogens [4, 5]. However, all IL-2 complex formulations are not similar in their mode of action. For example, complexes formed by conjugating recombinant IL-2 with the JES clone of anti-IL-2 mAb (clone JES6-5H4) stimulates the expansion of regulatory CD4⁺ T cells (Treg) [6]. This approach proved valuable for therapy against some experimental autoimmune diseases as well as the control of a mouse model for asthma [7, 8]. When complexes were formed between recombinant IL-2 with anti-IL-2 (clone S4B6) there was no effect on Treg, but instead the numbers and activity of NK cells and CD8⁺ T cells were enhanced [3, 6]. This approach has not been evaluated as a therapy against cutaneous infections such as those induced by herpes simplex virus (HSV) infection.

In the case of HSV infection in humans, cutaneous lesions occur either as a consequence of primary infection or reactivation from latent infection in the sensory nerve ganglia [9, 10]. Control of reactivated infection is mainly a function of CD8⁺ T cells [11, 12, 13, 14] and it is possible that lesions would be less likely to become clinically relevant if the CD8⁺ T cell responses were expanded. Testing such ideas are difficult to achieve in mouse model systems since mice with latent infections very rarely express clinical lesions even when latent virus does reactivate [15]. There is one mouse model however that does involve both cutaneous infection and spread of virus in the nervous system. This is the zosteriform model wherein virus spreads from the primary site of dermal infection to the sensory nerve ganglia where it replicates and disseminates throughout the dermatome to form a lesion, which in fact resembles shingles lesions caused by varicella zoster virus [16, 17]. This system provides a useful means of assessing the potential value of IL-2 complex therapy as we describe in this report. We showed that treatment with IL-2 complex starting day 1 after HSV-1 infection reduced lesion severity and virus burden in the skin when compared with their isotype IgG antibody treated counterparts. More detailed studies to define mechanisms of immunity were done in a footpad model where IL-2 complex treatment was started at day 1 post infection with HSV-1. The administration of IL-2 complex efficiently expanded HSV-1-gB-specific CD8⁺ T cells, increased the numbers of IFN- γ and TNF- α producing CD8⁺ T cells as well as reduced virus levels in mice infected with HSV-1 via the footpad route. CD8⁺ T cells in IL-2 complex treated animals had increased functionality as shown by increased expression of granzyme B, lytic granule release and increased the numbers of poly functional CD8⁺ T cells. Collectively, our results demonstrate that treatment with IL-2 complex provides a useful way to enhance protective immune responses against localized infections.

2. Materials & Methods

2.1 Mice and Virus

Five to six week old female C57BL6 mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The animals were housed in American Association of Laboratory Animal Care approved facilities at the University of Tennessee, Knoxville. All investigations followed guidelines of the Institutional Animal Care and Use Committee. HSV-1 KOS strain

was used in footpad and zosteriform model of infection. Virus was grown in Vero cell monolayers (American Type Culture Collection, Manassas, VA), titrated, and stored in aliquots at -80°C until used.

2.2 IL-2 Complex administration

IL-2 complex was formed by incubating $1.5\mu\text{g}$ of recombinant mouse IL-2 (eBioscience, CA) with $15\mu\text{g}$ anti-IL-2 Ab (S4B6) (BD biosciences, CA) for 15 minutes at room temperature as described before [18]. Each mouse was given i.p injection containing $1.5\mu\text{g}$ of recombinant mouse IL-2 complexed with $15\mu\text{g}$ anti-IL-2 mAb (S4B6) in $200\mu\text{l}$ of PBS on days 1, 2 and 3 post infection with HSV-1.

2.3 Footpad Infection with HSV-1

Infections of all mouse groups (6–8 week old) were conducted under a deep anesthesia (tribromoethanol). Mice were then injected subcutaneously in each hind footpad with 2×10^5 PFU HSV-1 KOS in $30\mu\text{l}$ of PBS. Each mouse was given i.p injection of the IL-2 complex on days 1, 2 and 3 post infection with HSV-1. Control mice were given $15\mu\text{g}$ rat IgG2a in PBS using the same schedule. Mice were sacrificed on day 6 post infection and the draining popliteal lymph nodes were isolated for analysis.

2.3.1 Zosteriform model of HSV-1 infection—Female C57BL/6 mice, 6 to 8 weeks of age were infected with HSV-1 KOS using the flank scarification skin infection model as previously described [16]. Briefly, mice were anesthetized (tribromoethanol) and the left flank was shaved and depilated with Nair. A small area of skin ($\sim 4\text{mm}^2$) near the top of the spleen was scarified 20 times using a 27-gauge needle and then the mice were infected topically by applying $10\mu\text{l}$ containing 1×10^6 PFU of the virus and a dressing was applied after 1 h for 24 h to facilitate infection [19]. Mice were injected i.p. with the IL-2 complex on days 1, 2 and 3 post infection with HSV-1. Control mice were given $15\mu\text{g}$ rat IgG2a in PBS. Mice were scored on different days post infection for skin lesion severity on a scale from 0 to 5: 0, no lesion; 1, redness or inflammation; 2, individual vesicle/lesion formation; 3, coalesced and/or ulcerated vesicles/lesions; 4, severe ulceration; and 5, necrosis. Mice were sacrificed 6 days post infection and the draining (axillary & brachial) lymph nodes on the left flank were isolated for analysis.

2.4 Quantification of virus in the footpad tissue and flank skin

Levels of infectious virus were determined as described previously [20]. Briefly, mice were sacrificed at day 6 post infection with HSV-1 and the footpad tissue was removed with a 11-gauge scalpel. Tissues were homogenized in 1 ml glass tissue grinders (Wheaton, Millville, NJ), centrifuged and the cell-free homogenate assayed at various dilutions on Vero cell monolayers in 12-well tissue culture plates overlaid with 1% methylcellulose. Plaques were visualized following fixation of the monolayers with 10% buffered formalin and staining with 0.5% crystal violet.

In the zosteriform infection, mice were sacrificed on day 6 post infection, a skin sample of $\sim 1\text{ cm} \times 2\text{ cm}$ (which includes site of inoculation and area of subsequent skin lesion) was

excised for analysis as described previously [21]. Titers of infectious virus in tissue homogenates were determined by standard plaque assay on Vero cells.

2.5 Antibodies and Reagents

Conjugated antibodies purchased from BD Bioscience were anti-CD8, anti-CD4, anti-CD69, anti-CD62L, anti-CD44, anti-CD122, anti-CD107a, anti-IFN- γ , anti-TNF- α and Ki-67. Granzyme B antibody was purchased from Life technologies. BrdU kit was purchased from BD biosciences. HSV gB₄₉₈₋₅₀₅ peptide (SSIEFARL) was supplied by Genscript. PE-Kb-gB tetramer was kindly provided by NIH tetramer core facility, Emory University, Atlanta, GA. The antibody-stained cells were acquired with a FACS Calibur (BD Biosciences) and the data were analyzed using the FlowJo software (Tree Star, OR).

2.6 Intracellular staining for IFN- γ and TNF- α

To enumerate the number of IFN- γ and TNF- α producing CD8⁺ T cells, intracellular cytokine staining was performed. In brief, 10⁶ freshly isolated lymph node cells were cultured in U bottom 96-well plates. Cells were left untreated or stimulated with SSIEFARL peptide (1 μ g/ml), and incubated for 5 h at 37°C in 5% CO₂. Brefeldin A (10 μ g/ml) was added for the duration of the culture period to facilitate intracellular cytokine accumulation. After this period, cell surface staining was performed, followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD biosciences) as per the manufacturer's recommendations.

2.6.1 Intracellular staining for granzyme B expression—To examine granzyme B expression in CD8⁺ T cells, intracellular cytokine staining was performed. Lymph node cells were surface stained with anti-CD8 and anti-CD44. This was followed by intracellular cytokine staining using a Cytofix/Cytoperm kit (BD biosciences). Anti-granzyme B or appropriate isotype control were used for intracellular staining.

2.6.2 Intracellular staining for Ki-67—For Ki-67 staining, single cell suspensions from draining lymph nodes were surface stained with anti-CD8, anti-CD44 and gB-tetramer. For intracellular Ki-67 staining the cells were fixed and permeabilized by incubating in cytofix/cytoperm solution (eBioscience) followed by staining with FITC-conjugated Ki-67 mAb (BD) or appropriate isotype control (BD).

2.7 BrdU treatment and detection

Mice were treated with 1 mg BrdU (BD biosciences) i.p. daily from day 1 to day 4 post infection. Mice were sacrificed on day 5 post infection and the draining lymph nodes were harvested for analysis. BrdU incorporation was assessed by the BrdU Flow kit (BD) per the manufacturer's instructions.

2.8 Degranulation Assay

To detect degranulation, draining lymph node cells were stimulated with gB peptide (1 μ g/ml) for 5h in a CO₂ incubator in the presence of brefeldin A (5 μ g/ml), monensin (5 μ g/ml) and FITC conjugated anti-CD107a was added at the beginning of the stimulation

period. Following stimulation, cells were washed and surface stained with anti-CD8, followed by intracellular cytokine staining for IFN- γ using a Cytotfix/Cytoperm kit (BD biosciences).

2.9 Statistical Analysis

Mann-Whitney test was used for statistical analyses. Prism 5.0 software (Graph pad, San Diego, CA) was used for analyses. $p < 0.05$ was considered as significant. Unless otherwise stated, plotted data represents mean $\pm$ standard error mean (s.e.m).

3. Results

3.1 IL-2 complex treatment reduced the lesion severity in the zosteriform model of HSV-1 infection

Formulations of IL-2 complexes formed by mixing recombinant IL-2 with anti-CD25 mAb (S4B6) were shown to selectively stimulate CD8⁺ T cells and control tumors in some mouse models [3]. To measure the outcome of cutaneous infection with HSV-1, the zosteriform model of HSV-1 infection was used. This model involves replication in both the skin and nervous system as also occurs in humans where virus reactivates from latency in the sensory ganglia to cause lesions in the innervated skin site [12]. The outcome of infection was compared in IgG (control) antibody treated animals to those treated with IL-2 complex on days 1, 2 and 3 post infection. Animals were followed for 6 days post infection to record lesion severity and on day 6, DLN were collected to enumerate HSV-gB-specific CD8⁺ T cells. The appearance of lesions was delayed in IL-2 complex treated animals and they showed reduced lesion scores compared to control mice (Fig. 1A). By day 6 post infection, 75% of control mice developed severe lesions (lesion score = 3) compared to only 12% percent in IL-2 complex treated mice (n=8). Furthermore, compared with control animals, IL-2 complex recipients showed 4 fold reduction in virus titers in the skin samples (Fig. 1B). Treatment of mice with IL-2 complex increased the numbers of HSV-1-gB specific CD8⁺ T cells and IFN- γ producing CD8⁺ T cells compared to isotype antibody treated control mice ($p = 0.02$) (Fig. 1C, D, E, F). These results show that IL-2 complex treatment reduced lesion severity, increased CD8⁺ T cell responses and provided enhanced protection against HSV-1 cutaneous infection.

3.2 Administration of IL-2 complex amplifies the CD8⁺ T cell responses following cutaneous footpad infection with HSV-1

To further define the immunological outcome of IL-2 complex therapy, a footpad model of infection was used which as many have shown results in readily detectable CD8⁺ T cell responses in the DLN [20, 22, 23]. Mice were infected in the hind footpads with HSV-1, and the infected mice were given IL-2 complex or rat IgG (control) on days 1, 2, 3 post infection. On day 6 post infection, mice treated with IL-2 complex showed a 4 fold reduction in HSV-1 levels in the footpad tissues compared to control mice (Fig. 2A). DLN were collected on day 6 post infection to enumerate and compare CD8⁺ T cell responses in IL-2 complex treated and control mice. Mice treated with IL-2 complex had significantly higher numbers of CD8⁺ T cells in the DLN compared with control mice (Fig. 2B). Analysis of Ki-67 expression, as a marker for proliferation *in vivo*, demonstrated that CD8⁺ T cells in IL-2 complex treated mice underwent extensive proliferation, with 85% of CD8⁺ T cells

showing Ki-67 expression compared to 24% in control mice (Fig. 2C, D). In addition, mice were given BrdU during the primary phase of the immune response to HSV-1 infection, showed a 3.5 fold increase in CD8⁺ T cells incorporating BrdU in IL-2 complex treated mice compared to control (Fig. 2 E, F). On average, 81% of CD8⁺ T cells isolated from the DLN of IL-2 complex treated mice incorporated BrdU (Fig. 2 F). In comparison, only 23% of CD8⁺ T cells incorporated BrdU in control mice. Flow cytometric analysis revealed a higher percentage of CD8⁺ T cells with an activated phenotype in the IL-2 complex treated animals compared to controls. There was a 2 fold increase in the frequency of CD8⁺ CD44^{hi} cell population in the IL-2 complex treated mice compared to controls (Fig. 3A, B). Furthermore, the majority of CD8⁺ T cells from IL-2 complex treated mice also expressed the early activation marker, CD69 (Fig. 3C). However, as described in a previous study [4], a higher proportion of activated CD8⁺ T cells from IL-2 complex treated mice were CD62L positive, indicative of a central memory phenotype [24] (Fig. 3D). Interestingly, a recent report shows that L-Selection/CD62L expression is important for the recruitment of activated CD8⁺ T cells to virus infected tissues for protective immunity [25]. In addition, a greater proportion of activated CD8⁺ T cells from IL-2 complex treated mice expressed CD122 (Fig. 3 E). This indicated that IL-2 complex treatment increases the frequencies of memory precursor CD8⁺ T cells. To determine the effect of IL-2 complex treatment on the expansion of HSV-1-specific CD8⁺ T cells, CD8⁺ T cells specific for the HSV-1-gB₄₉₈₋₅₀₅ epitope were quantified using a MHC class I tetramer. IL-2 complex treatment resulted in a significant increase in the numbers of HSV-1-gB-specific CD8⁺ T cell numbers compared to control mice (p 0.007) (Fig. 4A, B). A higher percentage of HSV-1-gB-specific CD8⁺ T cells in IL-2 complex treated mice expressed the proliferation marker, Ki-67 and activation marker, CD44 (Fig. 4C, D). Taken together, these observations demonstrate that IL-2 complex treatment promotes rapid expansion and activation of CD8⁺ T cells.

3.3 IL-2 complex treatment enhanced the functional capacity of CD8⁺ T cells following footpad infection with HSV-1

IL-2 complex administration resulted in a 2 fold increase in the numbers of IFN- γ producing CD8⁺ T cells after *in vitro* stimulation with gB-peptide (p 0.002) (Fig. 5A, B). The numbers of TNF- α producing CD8⁺ T cells were significantly higher in IL-2 complex treated mice compared to control mice (p 0.01) (Fig. 5C, D). In particular, IL-2 complex administration increased the proportion of CD8⁺ T cells that co-produced both IFN- γ and TNF- α (Fig. 5E), indicative of higher function. Also, CD8⁺ T cells from IL-2 complex treated animals had a higher frequency of cells that expressed granzyme B, necessary for cytolytic function [26]. On average, 27% of CD8 cells expressed granzyme B in IL-2 complex treated mice (Fig. 6A, B, C). In contrast, only 6% of CD8⁺ T cells expressed granzyme B in control mice. Granzyme B was undetectable in CD8⁺ T cells isolated from naïve mice, which is consistent with studies by others [27]. As an additional indicator of better function, more cells from IL-2 complex treated animals expressed the degranulation marker CD107a following *in vitro* stimulation of DLN cells with the gB peptide (Fig. 6D, E). These results indicate that IL-2 complex treatment increases the functionality of virus specific CD8⁺ T cells responses during HSV-1 infection.

4. Discussion

For many virus infections T cells, particularly CD8⁺ T cells, play a critical role in resolving infection [28]. When the response is of sufficient magnitude and functional activity, infections can be resolved promptly and lesions may be minimal. Thus one approach to reduce the consequences of infections is to boost the efficacy of CD8⁺ T cell responses. In the present report, we have evaluated an approach shown mainly in tumor systems to enhance CD8⁺ T cell immunity for its ability to reduce the expression of lesions caused by cutaneous infection by HSV-1 in mice. We were able to show using a zosteriform model in which a cutaneous infection results in skin lesions following passage to and replication within the local nerve ganglia [16], that animals treated post infection with IL-2 complex, had lesions that were delayed and less severe compared to animals treated with control IgG antibody. The therapeutic outcome was shown to correlate with an enhanced CD8⁺ T cell response in IL-2 complex treated animals. In addition, further characterization of the CD8⁺ T cell response in IL-2 complex treated animals was performed in a footpad infection model. It was shown that IL-2 complex recipients had virus specific CD8⁺ T cell responses that were significantly elevated in magnitude as well as in functional activity. Evidence for the later included the presence of a higher proportion of CD8⁺ T cells producing IFN- γ and TNF- α in IL-2 complex treated mice. In addition IL-2 complex recipients showed enhanced expression of granzyme B and increased lytic granule exocytosis in CD8⁺ T cells, critical for the expression of CD8⁺ T cell cytotoxic function [29]. Conceivably, the increase in IFN- γ and granzyme B expressing CD8⁺ T cells after IL-2 complex treatment might explain the reduced virus titers in the skin and consequently reduced lesion severity in mice treated with IL-2 complexes as both are known to be essential components required for virus clearance and preventing HSV reactivation from latency in neuronal tissues [30, 31].

The rationale for our experiments was at least in part based on research done on HSV in its natural host where recurrent lesions in the skin appear to be controlled largely by the CD8⁺ T cells [12]. Moreover, intriguingly, CD8⁺ T cells can be demonstrated as present at the nerve endings at the site where virus emerges following its reactivation from latency in the innervating ganglia [11]. Not all instances of reactivation result in lesions, perhaps because of the varying efficacy of the local T response [32, 33]. Several studies in the murine models also highlight the importance of CD8⁺ T cells in controlling HSV-1 infection and preventing reactivation [31, 34, 35]. We chose to evaluate the IL-2 complex approach since it proved effective in some cancer models to enhance CD8⁺ T cell responses, where CD8⁺ T cells are necessary for immunity [36]. However, limited studies have looked at pathogens and none have evaluated the effect of IL-2 complex against cutaneous virus infection. One study used IL-2 complexes given prophylactically, to enhance protection against *L. monocytogenes* or vaccinia virus [4]. Another study using MHV-68 as a chronic infection model showed that IL-2 complex therapy during the memory phase of infection reduced the persistent virus burden [5].

In previous studies, as well as those reported herein, the outcome of the IL-2 complex approach was to expand and activate the CD8⁺ T cell response. In our studies, this was sufficient to significantly reduce the expression of lesions and controlled the extent of virus replication. It would be interesting to evaluate if the approach might succeed in humans

where recurrent lesions are highly troublesome to many individuals. However if the approach could be used on an ethical basis, it would be desirable if the outcome remained durable. However, this situation is difficult to evaluate in mouse models other than comparing the effects on long term memory responses, since clinical recurrent responses are extremely difficult to attain. However, experiments are planned using a mouse ocular reoccurrence system to study the effects of IL-2 complex therapy on HSV ocular reactivation and lesion severity [37].

Although our studies emphasize that the efficacy of the IL-2 complex therapy approach succeeded by its effects on enhancing CD8⁺ T cell numbers and function, other mechanistic explanations could not be excluded. Notably, CD4⁺ T cells and NK cells are known to play an important role in the control of HSV-1 infection [38, 39]. Two recent studies have shown that IL-2/S4B6 immune complexes promote the expansion of NK cells in cancer as well as in a bacterial infection model [3, 4]. However, the effect of IL-2 complex therapy on CD4⁺ T cells and NK cells during HSV-1 infection is not known and needs further investigation.

One potential concern of IL-2 complex therapy is the effect on Treg. In fact some formulations of IL-2 and anti-IL-2 (clone JES6-5H4) do indeed enhance Treg numbers and function and such an approach has been advocated as a measure to control some autoimmune diseases as well as for HSV induced corneal immunopathology [7, 40]. However the formulation of IL-2 complex we used had no effect on numbers of Treg (data not shown) and these observations are consistent with another study [6]. Most probably, the outcome of treatment with IL-2 complexes is related to the selective binding of IL-2 mAb (clone S4B6 or JES-5H4) to specific sites on IL-2 which either promotes CD8⁺ T cell expansion or Treg expansion [41, 42].

In conclusion, it is conceivable that IL-2 complex therapy could be a useful approach to control the severity of cutaneous HSV-1 lesions.

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Figure 1. Treatment with IL-2 complex reduced lesion severity and enhanced CD8⁺ T cell responses in zosteriform model of HSV-1 infection

C57BL/6 mice were infected with HSV-1 KOS using flank scarification and the mice were observed for the development of lesions till day 6 pi. (A) Lesion severity was determined using the scoring system described in materials and methods. Lesion severity on different days post infection is shown (B) Skin samples were taken on day 6pi and virus titers were determined using standard plaque assay. Each circle or square represents an individual mouse and the horizontal bar represents mean (n=8 mice/group). Mice were sacrificed on day 6pi and draining axillary & brachial lymph nodes isolated from the left flank were homogenized and the cell suspensions were stained with different markers (C) Representative histogram plot showing proportion of CD8⁺ gB-tetramer⁺ T cells in the DLN (D) Total number of CD8⁺ gB-tetramer⁺ cells in the DLN, N=4 mice/group (E) Representative histogram plot showing CD8⁺ IFN- γ ⁺ T cells in the DLN, N=4 mice/group (F) Total numbers of CD8⁺IFN- γ ⁺ in the DLN. Data are representative of 3 independent experiments with 4 mice/group in each experiment. Data was analyzed using Mann Whitney test and are presented as mean $\pm$ S.E.M. $p < 0.05$ is reported as significant.

Figure 2. IL-2 complex treatment amplified CD8⁺ T cell proliferation following footpad infection with HSV-1

C57BL/6 mice were infected in each hind foot pad with 2×10^5 PFU HSV-1 KOS. Mice were sacrificed on 6 day p.i. at the peak of the immune response and draining popliteal lymph nodes were isolated for flow cytometric analysis. (A) Skin tissue was removed from the footpad and analyzed using viral plaque assay to determine the virus titer. The results are expressed as number of plaque forming unit of virus per each footpad tissue and the horizontal bar represents mean (n=8 mice/group). Cells isolated from PLN were stained with CD8, Ki-67 and BrdU markers. (B) Total number of CD8⁺ cells per PLN, n=7 mice/group (C) Representative histogram plot showing CD8⁺ Ki-67⁺ T cells. (D) Proportion of CD8⁺ Ki-67⁺ T cells in the PLN, n=4 mice/group (E) Representative histogram plot showing CD8⁺ BrdU⁺ T cells. (F) Proportion of CD8⁺ BrdU⁺ T cells in the PLN. F, n=4 mice/group. All

plots were gated on CD8⁺ T cells. Data was analyzed using Mann Whitney test and are presented as mean $\pm$ S.E.M. $p < 0.05$ was considered as significant. Experiments were repeated at least 3 times.

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Figure 3. IL-2 complex treatment increased activation of CD8⁺ T cells following footpad infection with HSV-1

Mice infected with HSV-1 were sacrificed on day 6 post-infection. Cells isolated from PLN were stained with CD8 and activation markers. (A) Representative histogram plot showing CD8⁺ CD44⁺ T cells (B) Percentage of CD8⁺ CD44^{hi} T cells in the PLN, n=4 mice/group (C) Representative histogram plot showing CD8⁺ CD69⁺ T cells (D) Representative histogram plot showing CD8⁺ CD44⁺ CD62L⁻ T cells (E) Representative histogram plot showing CD8⁺ CD44⁺ CD122⁺ T cells. All plots were gated on CD8⁺ T cells. Data was analyzed using Mann Whitney test and are presented as mean $\pm$ S.E.M. p<0.05 is reported as significant. Experiments were repeated at least 3 times.

Figure 4. IL-2 complex treatment enhanced the numbers of virus specific CD8⁺ T cells following footpad infection with HSV-1

Mice infected with HSV-1 were sacrificed on day 6 post-infection and PLN cells were stained with gB₄₉₈₋₅₀₅-tetramer to identify virus specific CD8⁺ T cells using flow cytometry. (A) Representative histogram plot showing proportion of CD8⁺ gB-tetramer⁺ T cells in the PLN. (B) Total number of CD8⁺ gB-tetramer⁺ cells in the PLN, n=7 mice/group (C) Representative histogram plot showing CD8⁺ gB-Tetramer⁺ Ki-67⁺ T cells (D) Representative histogram plot showing CD8⁺ gB-Tetramer⁺ CD44⁺ T cells in the PLN. All plots were gated on CD8⁺ T cells. Data was analyzed using Mann Whitney test and are presented as mean ± S.E.M. p<0.05 was considered as significant. Experiments were repeated at least 3 times.

Figure 5. IL-2 complex treatment increased the functional capacity of CD8⁺ T cells following footpad infection with HSV-1

Mice infected with HSV-1 were sacrificed on day 6 post-infection. Single cell suspensions obtained from PLN were stimulated with the immunodominant gB (SSIEFARL) peptide and cytokine producing CD8⁺ T cells were determined by flow cytometry as described in the methods. (A) Representative histogram plot showing CD8⁺ IFN- γ ⁺ T cells in the PLN. (B) Total numbers of CD8⁺ IFN- γ ⁺ T cells in the PLN, n=7 mice/group (C) Representative histogram plot showing CD8⁺ TNF- α ⁺ T cells in the PLN (D) Total numbers of CD8⁺ TNF- α ⁺ T cells in the PLN, n=7 mice/group (E) Representative histogram plot showing the percentage of CD8⁺ T cells capable of producing both IFN- γ and TNF- α . All plots were gated on CD8⁺ T cells. Data was analyzed using Mann Whitney test and are presented as mean $\pm$ S.E.M. p<0.05 is reported was considered as significant. Experiments were repeated at least 3 times.

Figure 6. IL-2 complex treatment enhanced granzyme B expression and increased lytic granule release in CD8⁺ T cells following footpad infection with HSV-1

Mice infected with HSV-1 were sacrificed on day 6 post-infection. Intracellular staining was performed on cells obtained from PLN and granzyme B expressing CD8⁺ T cells were analyzed using flow cytometry as described in the methods (A) Representative histogram plot showing expression of granzyme B on CD8⁺ T cells in the PLN. (B) Representative plot showing CD8⁺ granzyme B⁺ T cells (C) Percentage of CD8⁺ granzyme B⁺ T cells in the PLN, (n=4 mice/group). D-E, Degranulation assay was performed on cells obtained from PLN as described in the materials and methods (D) Representative histogram plot showing CD8⁺ CD107a⁺ T cells. (E) Total numbers of CD8⁺ CD107a⁺ T cells in the PLN (n=4 mice/group). All plots were gated on CD8⁺ T cells. Data was analyzed using Mann Whitney test and are presented as mean $\pm$ S.E.M. $p < 0.05$ was considered as significant. Experiments were repeated at least 2 times.