EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 37, No. 3, 193-198, June 2005

In vivo ligation of glucocorticoid-induced TNF receptor enhances the T-cell immunity to herpes simplex virus type 1

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Accepted 6 May 2005

Abbreviations: DLN, draining lymph node; gB, glycoprotein B; gD, glycoprotein D; GITR, glucocorticoid-induced tumor necrosis factor receptor; GVHD, graft-versus-host disease; HSV-1, herpes simplex virus type 1

Abstract

GITR (glucocorticoid-induced TNF receptor) is a recently identified member of the TNF receptor superfamily. The receptor is preferentially expressed on $CD4^+CD25^+$ regulatory T cells and GITR signals break the suppressive activity of the subset. In this study, we wanted to reveal the *in vivo* function of GITR in herpes simplex virus type 1 (HSV-1) infection. A single injection of anti-GITR mAb (DTA-1) immediately after viral infection significantly increased the number of $CD4^+$ and $CD8^+$ T cells expressing CD25, an activation surface marker, and secreting IFN-γ. We confirmed these *in vivo* observations by showing *ex vivo* that re-stimulation of $CD4^+$ or $CD8^+$ T cells with a $CD4^+$ or $CD8^+$ T-cell-specific HSV-1 peptide, respectively, induced a significant elevation in cell proliferation and in IFN-γ secretion. Our results indicate that GITR signals play a critical role in the T-cell immunity to HSV-1.

Keywords: GITR; HSV-1; gB; gD; Th1 cell; CTL

Introduction

GITR (glucocorticoid-induced TNF receptor) was originally identified by comparing untreated and dexamethasone-treated murine T-cell hybridoma cells (Nocentini *et al.,* 1997). Later, human orthologue and its ligand were cloned by searching an EST (expressed sequence tag) database (Gurney *et al.,* 1999; Kwon *et al.,* 1999a; 1999b; 2003). GITR expression is up-regulated on T cells after activation, and a high level of GITR is constitutively expressed on $CD4^+$ $CD25^+$ regulatory T cells (McHugh *et al.,* 2002; Shimizu *et al.,* 2002). Initial characterization of GITR functions revealed that the receptor could inhibit T-cell receptor (TCR)-induced apoptosis in the T cell hybridoma that was used to clone the GITR gene (Nocentini *et al.,* 1997). This was confirmed in a human T cell line (Gurney *et al.,* 1999). Consistently, GITR-deficient mouse T cells exhibited a higher capacity to proliferate in response to TCR stimulation but underwent higher levels of activation-induced cell death (Ronchetti *et al.,* 2002). In $CD4^+CD25^+$ regulatory T cells, GITR signals have been shown to abrogate their suppressive function (McHugh *et al.,* 2002; Shimizu *et al.,* 2002). However, recent studies have demonstrated that GITR functions as a co-stimulatory molecule for conventional T cells *in vitro* (Ji *et al.,* 2004; Kanamaru *et al.,* 2004; Kohm *et al.,* 2004; Muriglan *et al.,* 2004; Ronchetti *et al.,* 2004) and that GITR-deficient mouse $CD4^+CD25^+$ regulatory T cells were able to exert the suppressive activity on conventional $CD4^+$ T cells as equally as wild-type $CD4^+CD25^+$ regulatory T cells (Ronchetti *et al.,* 2002).

The *in vivo* function of GITR remains largely to be defined. In this study, we wanted to reveal the *in vivo* function of GITR in an HSV-1 infection model. Our data demonstrated that ligation of GITR markedly increased the T-cell immunity to HSV-1.

Materials and Methods

Mice and peptides

Female Balb/c mice, 6-8 wk of age, were purchased from Orient (Seoul, Korea). The HSV-1 glycoprotein $D_{246-261}$ ($gD_{246-261}$: APYTSTLLPPELSETP) peptide was described previously (Grammer *et al.,* 1990) and was shown to be specific for Th1 cells (BenMohamed *et al.,* 2003). The $gB_{498-505}$ (SSIEFARL) peptide is an immunodominant peptide for $CD8^+$ T cells (Hanke *et al.,* Witmer *et al.,* 1990; Bonneau *et al.,* 1993). The peptides were synthesized by Peptron (Daegen, Korea).

mAbs and flow cytometry

Anti-GITR mAb (DTA-1) was described previously (Shimizu *et al.,* 2002) and purified from ascites. Control rat Ig was purchased from Sigma (St Louis, MO). FITC-anti-CD3, FITC- or phycoerythrin (PE)-anti-

CD4, FITC- or PE-anti-CD8, PE-anti-CD25, and PE-anti-IFN-γ mAbs were purchased from BD Biosciences Pharmingen (San Diego, CA). Lymphocytes were stained and analyzed as described previously (Kim *et al.,* 2005). Intracellular staining for IFN-γ was performed according to the manufacturer's instruction (BD Biosciences Pharmingen).

Virus and infection

The KOS strain of HSV-1 was used in this study. For the production of the virus stock supernatant, the virus-infected Vero cell cultures were centrifuged at 1,500 rpm and filtered with a 0.45-μm mesh filter to remove cellular debris. The viruses were titrated using the end-point dilution method. Balb/c mice were inoculated with 5×10^5 PFU of HSV-1 injected s.c. into each hind footpad. DTA-1 or control Ig (300 μg per mouse) was i.p. injected immediately thereafter.

Proliferation assay

The DLNs were harvested from HSV-1-infected mice and a single cell suspension was prepared in PBS. The cell debris was removed by passing through 70-μm cell strainer (BD Falcon, San Diego, CA). Isolated lymphocytes (5×10^5) were cultured at various concentrations of peptide for 72 h. Proliferation was assessed in triplicate by pulsing with 1 Ci[^{3}H]thymidine for 12 h.

ELISA

IFN-γ was measured from culture supernatant, using an ELISA kit (Endogen, Woburn, MA), according to the manufacturer's protocol.

Statistical analysis

Student *t* test was used to determine the statistical significance of differences between experimental groups.

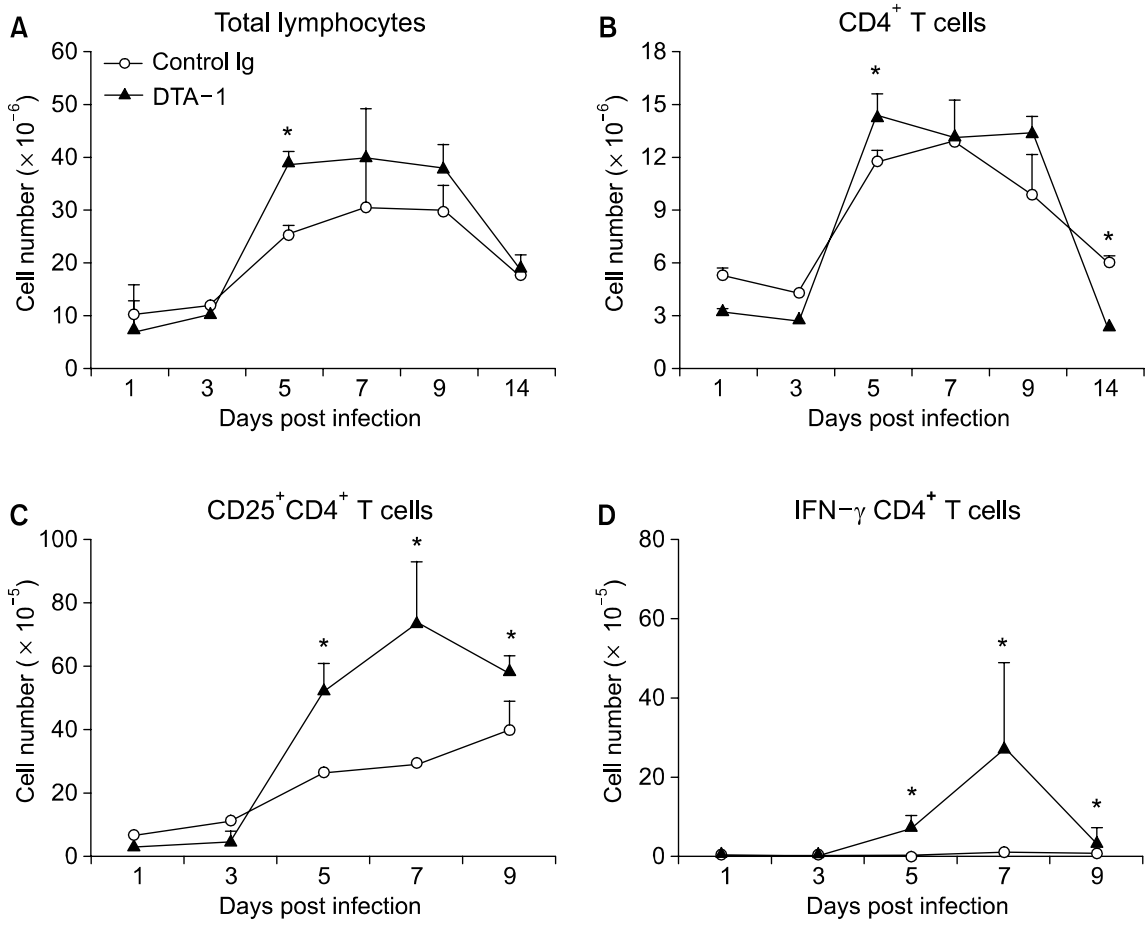

Figure 1. Ligation of GITR activates CD4$^+$ T cells after HSV-1 infection. Mice were infected in the hind foodtpad with 5×10^5 PFU of HSV-1. DTA-1 or control Ig (300 μg per mouse) was i.p. injected immediately thereafter. (A) The number of total lymphocytes isolated from the DLN. (B) CD4$^+$ T cells derived from the DLN were counted by staining the DLN cells with anti-CD3 and anti-CD4. (C) Activated CD4$^+$ T cells were counted by staining the DLN cells with anti-CD4 and anti-CD25. (D) The DLN cells were stained for CD4 combined with intracellular staining for IFN-γ. Data are shown as mean ± SD of $n = 3$. *$P < 0.05$, between the 2 groups at the indicated time points.

Results and Discussion

Agonistic anti-GITR antibody (DTA-1) has been shown to abolish the immunosuppressive activity of $CD4^+$ $CD25^+$ regulatory T cells (Shimizu *et al.,* 2002). Administration of DTA-1 exacerbates experimental autoimmune encephalomyelitis (Kohm *et al.,* 2004) and acute graft-versus-host disease (GVHD) (Muriglan *et al.,* 2004). Using this agonistic mAb, we examined its effect on the immune response to HSV-1 infection. The kinetics for the expansion of total lymphocytes and $CD4^+$ T cells was similar in the DLN of DTA-1- and control Ig-treated mice at all the time points investigated except for 5-day postinfection (Figure 1A and B). We next counted activated $CD4^+$ T cells by analyzing the expression of CD25 on the cell surface of $CD4^+$ T cells and by staining intracellular IFN-γ that represents effector T cells more precisely. Both methods showed that a noticeable expansion of activated $CD4^+$ T cells from DTA-1-treated mice began at 5-day postinfection and reached a peak at day 7 (Figure 1C and D). In comparison with control Ig-treated mice, marked increase in activated $CD4^+$ T-cell numbers was observed in DTA-1-treated mice between day 5 and day 9 after infection (Figure 1C and D). Although activated $CD4^+$ T cells expressing CD25 existed in the DLN of control Ig-treated mice, production of intracellular IFN-γ in $CD4^+$ T cells was barely detectable (Figure 1D). Our results indicate that DTA-1 potently induced the expansion of effector $CD4^+$ T cells to viral antigens.

Unlike $CD4^+$ T cells, $CD8^+$ T-cell numbers were significantly greater between day 5 and day 9 in the DLN of DTA-1-treated mice compared with those of control Ig-treated mice (Figure 2A). At day 14, the number of both $CD4^+$ and $CD8^+$ T cells of DTA-1-treated mice was significantly reduced, presumably as a result of activation-induced death (Figure 1A and 2A). The kinetics of activated CD8+ T-cell expansion was parallel to that of activated CD4+ T-cell expansion (Figure 2C and D). We did not detect activation of $CD4^+$ and $CD8^+$ T cells in non-draining lymph nodes in either group after HSV-1 infection (data not shown). Taken together, our results suggest that *in vivo* ligation of GITR can significantly increase activation of both $CD4^+$ and $CD8^+$ T cells after HSV-1 infection.

To directly verify whether ligation of GITR induces the generation of antigen-specific T cells, the DLN-derived T-cell responses to peptide epitopes were examined *ex vivo* 7 days after infection. Our results showed that the Th1-specific $gD_{246\text{-}261}$ peptide vigo-

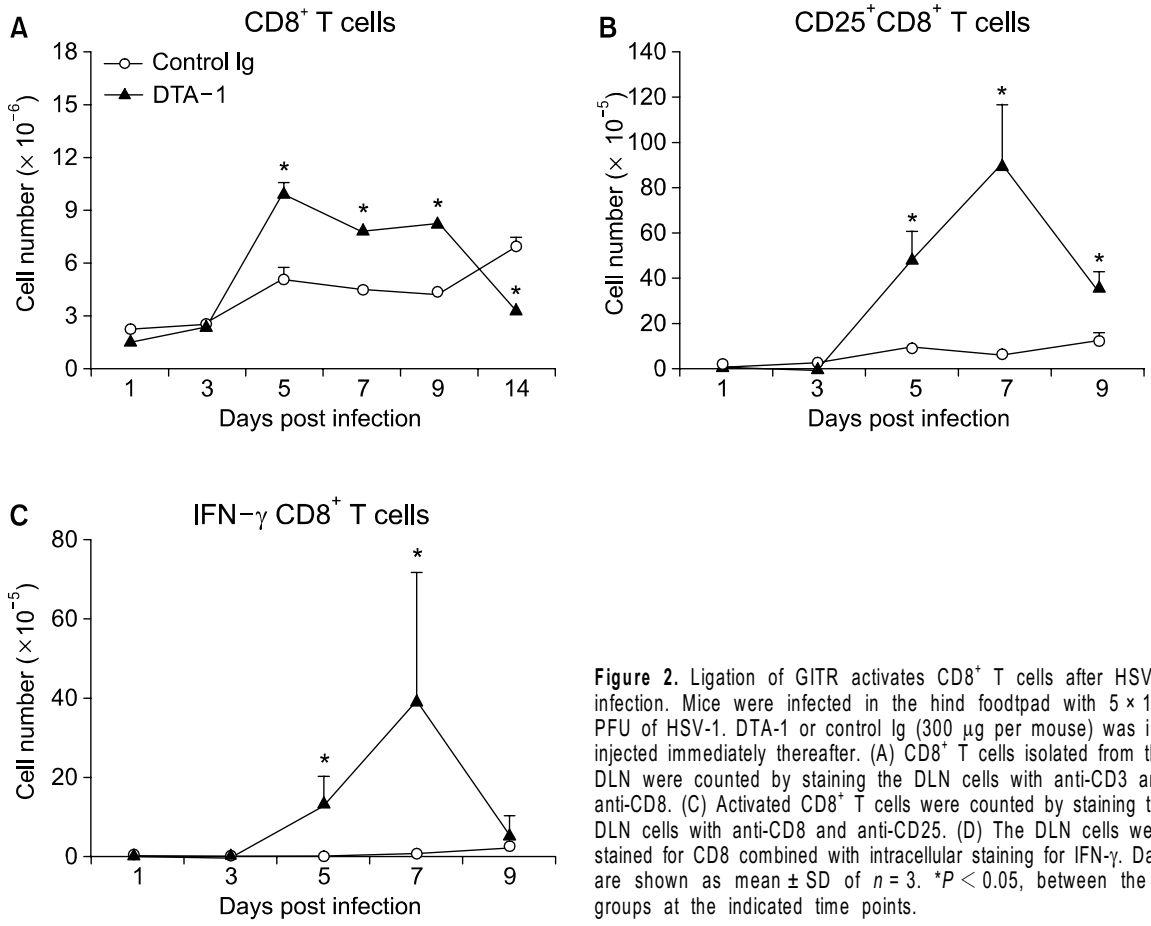

Figure 2. Ligation of GITR activates $CD8^+$ T cells after HSV-1 infection. Mice were infected in the hind foodtpad with 5×10^5 PFU of HSV-1. DTA-1 or control Ig (300 μg per mouse) was i.p. injected immediately thereafter. (A) $CD8^+$ T cells isolated from the DLN were counted by staining the DLN cells with anti-CD3 and anti-CD8. (C) Activated $CD8^+$ T cells were counted by staining the DLN cells with anti-CD8 and anti-CD25. (D) The DLN cells were stained for CD8 combined with intracellular staining for IFN-γ. Data are shown as mean ± SD of $n = 3$. $^*P < 0.05$, between the 2 groups at the indicated time points.

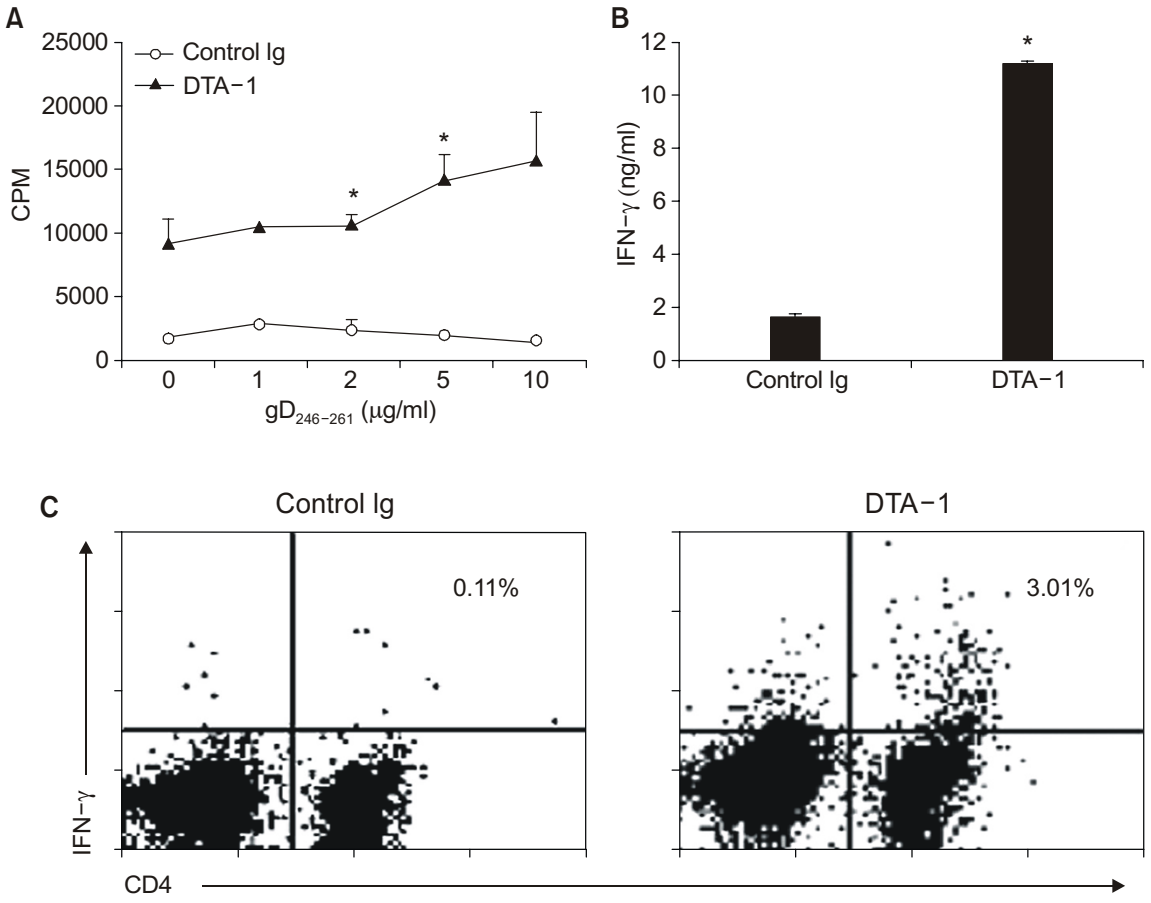

Figure 3. Increase in $gD_{246-261}$-specific T cells. (A) The cells isolated from the DLN at 7-day postinfection were stimulated with various concentrations of $gD_{246-261}$ peptide for 72 h. Proliferation was assessed in triplicate by pulsing with 1 Ci[^{3}H]thymidine for the last 12 h. (B) After the DLN cells were stimulated with 10 μg of $gD_{246-261}$ peptide for 24 h, supernatants were collected and assayed for IFN-γ by ELISA. (C) The DLN cells were stimulated with 10 μg of $gD_{246-261}$ peptide for 6 h and were stained for CD4 combined with intracellular staining for IFN-γ. $*P < 0.05$, between the 2 groups.

rously stimulated the proliferation of T cells from DTA-1-treated mice in a dosage-dependent manner (Figure 3A). In our experimental conditions, T cells of control Ig-treated mice had a minimal proliferation. In consistence, peptide-stimulated T cells of DTA-1-treated mice secreted high levels of IFN-γ compared with those of control Ig-treated mice (Figure 3B). Intracellar IFN-γ staining analysis demonstrated that DTA-1-treated mice had approximately 30-fold increase in the frequency of $gD_{246-261}$ peptide-specific $CD4^+$ T cells as compared with control Ig-treated mice (Figure 3C). Similarly, the $CD8^+$ T cell-specific $gB_{498-505}$ peptide induced such a vigorous *in vitro* response as the $gD_{246-261}$ peptide did (Figure 4). These data indicate that *in vivo* GITR stimulation can augment the expansion of antigen-specific $CD4^+$ and $CD8^+$ T cells after HSV-1 infection.

The expression pattern of GITR suggests that GITR may be involved in functions of a variety of immune cells, including T cells, B cells and macrophages (Shimizu *et al.,* 2002). In agreement of our data presented in this report, results from other *in vivo* studies have shown that GITR stimulation increases Th1 autoreactivity in experimental autoimmune encephalomyelitis (Kohm *et al.,* 2004) and $CD8^+$ T cell-mediated acute GVHD (Muriglan *et al.,* 2004). Ligation of GITR also has been shown to markedly enhance the $CD8^+$ T-cell activity in chronic GVHD (manuscript in submission). It is possible that several mechanisms of action underlying the *in vivo* observations are operative; GITR signals either activate the $CD4^+$ and $CD8^+$ T cells through depressing the immunosuppressive activity of $CD4^+CD25^+$ T cells (Kohm *et al.,* 2004) or directly activate $CD25^-CD4^+$ or $CD25^-CD8^+$ conventional T cells (Muriglan *et al.,* 2004; manuscript in submission). In a chronic GVHD that is mediated by Th2 cells, *in vivo* engagement of GITR prevents donor $CD8^+$ T-cell tolerance and the subsequent activation of donor $CD8^+$ T cells and Th1 $CD4^+$ T cells shifts chronic GVHD toward acute GVHD (manuscript in submission).

The main finding presented in this report is to

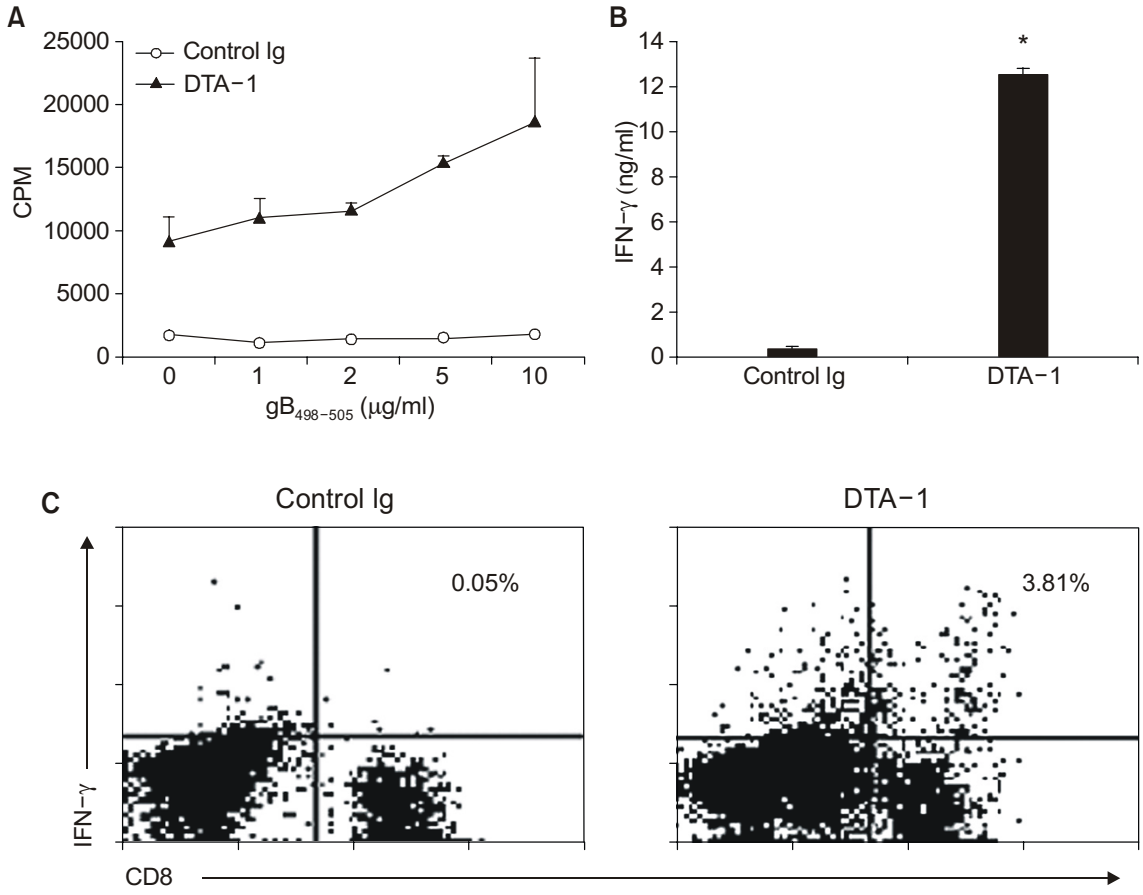

Figure 4. Increase in $gB_{498-505}$-specific T cells. (A) The cells isolated from the DLN at 7-day postinfection were stimulated with various concentrations of $gB_{498-505}$ peptide for 72 h. Proliferation was assessed in triplicate by pulsing with 1 Ci[^{3}H]thymidine for the last 12 h. (B) After the DLN cells were stimulated with 10 μg of $gB_{498-505}$ peptide for 24 h, supernatants were collected and assayed for IFN--γ by ELISA. (C) The draining lymph node cells were stimulated with 10 μg of $gB_{498-505}$ peptide for 6 h and were stained for CD8 combined with intracellular staining for IFN-γ. *$P < 0.05$, between the 2 groups.

reveal *in vivo* the involvement of a GITR co-stimulatory pathway in viral infection. To be equally important, we provide a possibility of GITR stimulation as an adjuvant in elevating the immune response to efficiently remove viral infection. Our results demonstrated that ligation of GITR induced approximately 30-fold and 80-fold increase in the frequency of antigen-specific $CD4^+$ Th1 cells and $CD8^+$ T cells, respectively (Figure 3 and 4). It seems that *in vivo* ligation of GITR removes HSV-1 viruses from the infection site rapidly (our preliminary data). Since the T-cell response takes an action against viruses 5-7 days after infection, the rapid kinetics for the viral removal indicates that GITR signals may be important for anti-viral activities mediated by other cells (*e.g.*, macrophages, dendritic cells, NK cells, and residential cells in the infection site) than T cells. Therefore, this adjuvant effect of anti-GITR mAb may be critical in removing viruses by triggering the innate immunity. In sum, our data suggest a possibility that *in vivo* ligation of GITR may be effective in removing the primary viral infection.

Acknowledgement

This work was supported by grants from University of Ulsan (2003-0087) and the SRC Fund to the IRC from KOSEF and the Korean Ministry of Science and Technology.

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