Opposite Roles of B7.1 and CD28 Costimulatory Molecules for Protective Immunity against HSV-2 Challenge in a gD DNA Vaccine Model

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ABSTRACT

Background: Costimulation is a critical process in Ag-specific immune responses. Both B7.1 and CD28 molecules have been reported to stimulate T cell responses during antigen presentation. Therefore, we tested whether Ag-specific immune responses as well as protective immunity are influenced by coinjecting with B7.1 and CD28 cDNAs in a mouse HSV-2 challenge model system. **Methods:** ELISA was used to detect levels of antibodies, cytokines and chemokines while thymidine incorporation assay was used to evaluate T cell proliferation levels. **Results:** Ag-specific antibody responses were enhanced by CD28 coinjection but not by B7.1 coinjection. Furthermore, CD28 coinjection increased IgG1 production to a significant level, as compared to pgD+ pcDNA3, suggesting that CD28 drives Th2 type responses. In contrast, B7.1 coinjection showed the opposite, suggesting a Th1 bias. B7.1 coinjection also enhanced Ag-specific Th cell proliferative responses as well as production of Th1 type cytokines and chemokines significantly higher than pgD+pcDNA3. However, CD28 coinjection decreased Ag-specific Th cell proliferative responses as well as production of Th1 types of cytokines and chemokine significantly lower than pgD+pcDNA3. Only MCP-1 production was enhanced by CD28. B7.1 coimmunized animals exhibited an enhanced survival rate as well as decreased herpetic lesion formation, as compared to pgD+ pcDNA3. In contrast, CD28 vaccinated animals exhibited decreased survival from lethal challenge. **Conclusion:** This study shows that B7.1 enhances protective Th1 type cellular immunity against HSV-2 challenge while CD28 drives a more detrimental Th2 type immunity against HSV-2 challenge, supporting an opposite role of B7.1 and CD28 in Ag-specific immune responses to a Th1 vs Th2 type. **(Immune Network 2005;5(2): 68-77)**

Key Words: Costimulatory molecules, cytokines, chemokines, infectious immunity-virus, Th1/Th2

Introduction

Antigen-specific T cell activation requires two independent signaling events, one mediated through T cell receptor (TCR) engagement with peptide/ major histocompatibility complex (MHC) Class II molecules on antigen presenting cells (APCs), and the second through the cognate interaction of costimulatory molecules expressed on T cells and APCs. Costimulatory molecules, CD80 (B7.1) and CD86 (B7.2) expressed on APCs deliver potent costimulatory signals through CD28/CTLA-4 on T cells which drive T cells into the cell cycle, induce differentiation and augment cytokine production for an effective immune response (1,2). Although B7.1 and B7.2 have a limited homology (28%), B7.1 and B7.2-expressing transfectants efficiently costimulate anti-CD3-induced T cell proliferation and cytokine production *in vitro* (3), suggesting that the contexts in which B7.1 or B7.2 operates are important to T cells during antigen presentation *in vivo*. In contrast, the CD28 glycoprotein is expressed as a homodimer on the surface of 95% of CD4+ T cells and about 50% of CD8+ T cells (4). It is known that CD28 costimulation activates Th2 promoting transcription factor, GATA-

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3, and increases IL-4 receptor sensitivity, resulting in promoting Th type 2 differentiation (5,6). More recently, it has been reported that CD28 costimulation induces both type 2 CD4+ and CD8+ T cell polarization (7) and that the CD28-driven immune cell stimulation is mediated via B7.1 and B7.2 (8). In pathogenic protection, CD28-deficient animals showed more severe parasitic infection resulting from defects in cellular immunity (9). In particular, DNA vaccine studies in the HIV, influenza, tuberculosis and HSV systems reported that antigen-specific cellular responses are enhanced significantly by co-delivery with B7.1 or B7.2 (10-13). However, no studies on CD28 have been reported.

Herpes simplex virus (HSV) is the causative agent of a spectrum of human diseases, such as cold sores, ocular infections, encephalitis and genital infections (14). It has been reported that Th1 type CD4+ T cells but not CD8+ T cells apparently play a crucial role for protection from HSV-2 challenge (15-18).

Using this defined model we tested specific roles of one of B7 molecules, B7.1 and CD28 in the induction of Ag-specific immune responses as well as protective immunity against HSV-2. We observe that B7.1 molecules drive a more potent Ag-specific Th1 type cellular responses, resulting in decreasing both mortality and morbidity due to HSV-2 infection. In contrast, CD28 induces Ag-specific immune responses towards a Th2 phenotype, resulting in making more susceptible for HSV-2 challenge.

Materials and Methods

Mice. Female 4 to 6 weeks old BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Their care was under the guidelines of the National Institutes of Health (Bethesda, MD) and the University of Pennsylvania IACUC (Philadelphia, PA).

Reagents. HSV-2 strain 186 (a kind gift from Dr. Schaffer of University of Pennsylvania, Philadelphia, PA) was propagated in the Vero cell line (ATCC, Rockville, MD). DNA vaccine (pgD) encoding HSV-2 gD protein was previously tested (17). The expression vector, pcDNA3-B7.1 was previously reported (11). CD28 genes were cloned from cDNA library using 2 primers (forward primer: 5'-GATATC ATGCTCAGGCTGCTCTTGGCT-3', reverse primer: 5'-CGGGATCCTCAGGAGCGATAGGCTGCG AA-3'). The introduced restriction endonuclease recognition sequences are shown in underline. The Eco RV-Bam HI fragment of CD28 genes was cloned into a pcDNA3.1 (−) vector (Invitrogen, Carlsbad, CA). The CD28 plasmid DNA was verified by DNA sequencing analysis. Plasmid DNA was produced in bacteria and purified by double banded CsCl preparations. Recombinant HSV-2 gD proteins, a generous gift from Drs. Cohen and Eisenberg of University of Pennsylvania, Philadelphia, PA, were used as recombinant antigen in these studies.

Expression of B7.1 and CD28 expression constructs. Expression of B7.1 and CD28 constructs was analyzed by transfecting them into RD cells as described previously (11). Cells were harvested 72 hrs after transfection and tested for expression using FACS analysis with FITC-conjugated monoclonal antibodies for B7.1 and CD28 (PharMingen, San Diego, CA).

DNA inoculation of mice. The quadriceps muscles of BALB/c mice were injected with pgD DNA constructs formulated in a final volume of 100 μl of PBS and 0.25% bupivacaine-HCl (Sigma, St. Louis, MO) using a 28-gauge needle (Becton Dickinson, Franklin Lakes, NJ). B7.1 and CD28 expression plasmid cassettes were mixed with pgD plasmid solution prior to injection.

ELISA. ELISA was performed as previously described (19,20). In particular, for the determination of relative levels of gD-specific IgG subclasses, anti-murine IgG1, or IgG2a conjugated with HRP (Zymed, San Francisco, CA) were substituted for anti-murine IgG-HRP. Furthermore, ELISA titers were determined as the reciprocal of the highest sera dilution showing the same OD value as sera of naive mice.

T helper (Th) cell proliferation assay. Th cell proliferation assay was performed as previously described (19,20).

Detection of Cytokines and Chemokines. A 1 ml aliquot containing 6×10^6 splenocytes was added to wells of 24 well plates. Then, 1 μg of HSV-2 gD protein/ml was added to each well. After 2 days incubation at 37°C in 5% CO_2, cell supernatants were secured and then used for detecting levels of IL-2, IL-10, IFN-γ, RANTES, MIP-1 α and MCP-1 using commercial cytokine and chemokine kits (Biosource, Intl., Camarillo, CA and R&D Systems, Minneapolis, MN) by adding the extracellular fluids to the cytokine or chemokine-specific ELISA plates.

Intravaginal HSV-2 challenge. After DNA injection, mice were challenged intravaginally (i.vag.) with HSV-2 strain 186 as previously described with some modifications (21,22). Before inoculating the virus, the intravaginal area was swabbed with a cotton tipped applicator (Hardwood Products Company, Guiford, ME) soaked with 0.1 M NaOH solution and then cleaned with dried cotton applicators. Mice were then examined over time to evaluate pathological conditions and survival rates.

Statistical analysis. Statistical analysis was done using independent Student's t test and Chi-square test. Values between different immunization groups were compared. p values <0.05 were considered significant.

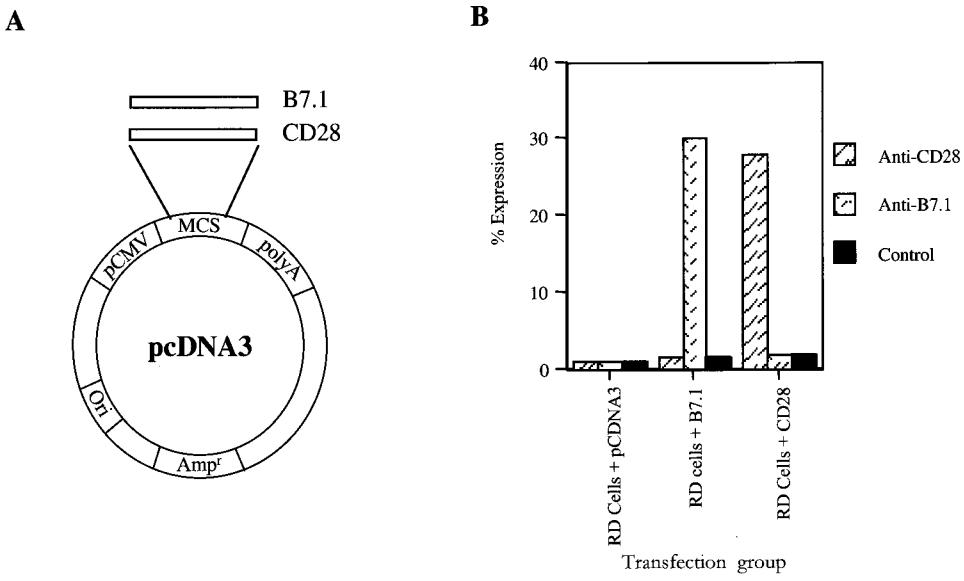

Figure 1. Construction (A) of pcDNA3-B7.1 and pcDNA3-CD28 and their expression (B) on human rhabdomyosarcoma (RD) cells *in vitro*. RD cells were transfected with pcDNA3 (control), pcDNA3-B7.1, or pcDNA3-CD28. Three days after transfection, the cells were removed from the plates and were analyzed by FACS analysis using either control antibodies, anti-B7.1, or anti-CD28 antibodies to detect expression of the transfected gene products. Percent expression represents FITC positive cell/total cells $\times 100$.

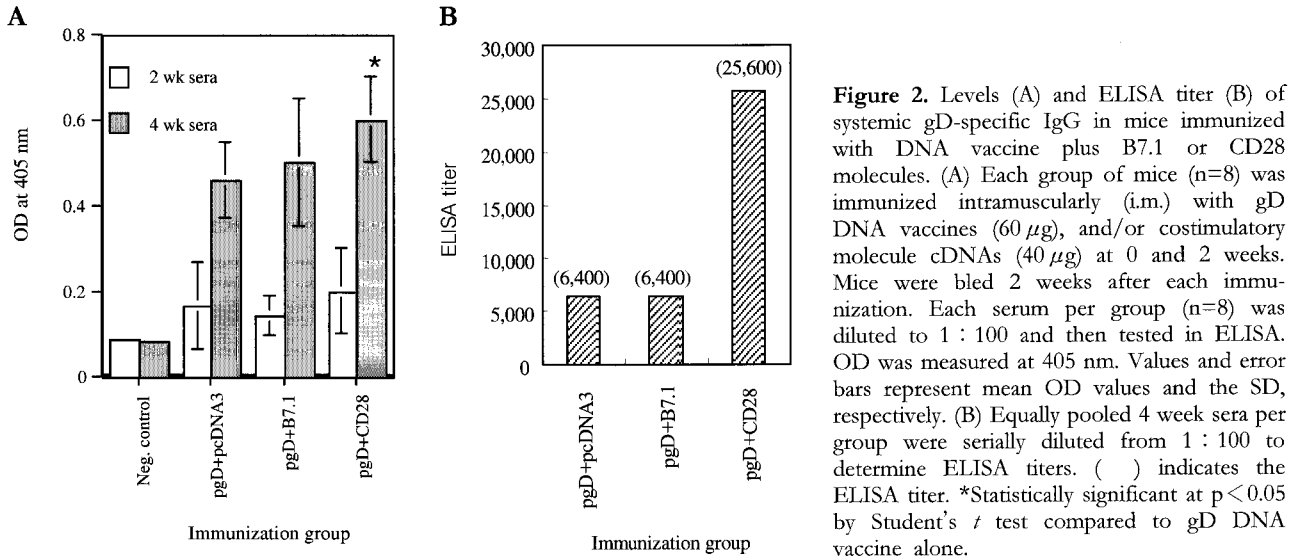

Figure 2. Levels (A) and ELISA titer (B) of systemic gD-specific IgG in mice immunized with DNA vaccine plus B7.1 or CD28 molecules. (A) Each group of mice (n=8) was immunized intramuscularly (i.m.) with gD DNA vaccines (60 μg), and/or costimulatory molecule cDNAs (40 μg) at 0 and 2 weeks. Mice were bled 2 weeks after each immunization. Each serum per group (n=8) was diluted to 1 : 100 and then tested in ELISA. OD was measured at 405 nm. Values and error bars represent mean OD values and the SD, respectively. (B) Equally pooled 4 week sera per group were serially diluted from 1 : 100 to determine ELISA titers. ( ) indicates the ELISA titer. *Statistically significant at $p < 0.05$ by Student's t test compared to gD DNA vaccine alone.

Results

In vitro expression of B7.1 and CD28 constructs. The genes for B7.1 and CD28 were individually cloned into a pcDNA3 backbone plasmid (Fig. 1). To test whether these constructs could express their relevant proteins, we transfected them into RD cells. Using FACS analysis, we observed that expression vectors, pcDNA3-B7.1 and pcDNA3-CD28 resulted in specific expression of B7.1 and CD28, respectively (Fig. 1).

Systemic antibody responses. To determine if coinjection of gD genetic vaccines with B7.1 and CD28 expression vectors might influence humoral immune responses against gD, each serum per group was tested by ELISA. As shown in Fig. 2A, gD-specific IgG levels were enhanced significantly by coinjection with CD28. In contrast, B7.1 coinjection showed the IgG production pattern similar to pgD+pcDNA3. ELISA titers of equally pooled 4 week sera were also determined to be 6,400 (pgD+pcDNA3), 6,400 (pgD+B7.1) and 25,600 (pgD+CD28) (Fig. 2B). Similar results were observed when we tested sera obtained 1 month after the initial immunization with gD DNA vaccine (10 μg per mouse) plus costimulatory molecules (40 μg per mouse) (data not shown). This shows that coinjection with B7.1 has little effect on antibody production, whereas CD28 coinjection has a positive effect on antibody production, suggesting a Th2 type bias of this codelivery.

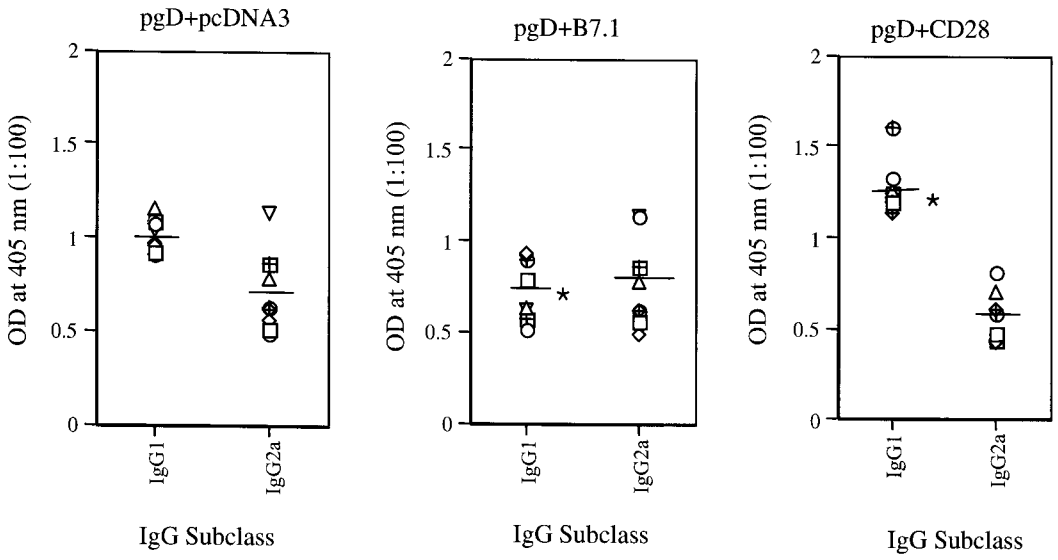

Figure 3. Levels of systemic gD-specific IgG1 and IgG2a in mice immunized with DNA vaccine plus B7.1 or CD28. Each group of mice (n=8) was immunized i.m. with gD DNA vaccines (60 μg), and/or costimulatory molecule cDNAs (40 μg) at 0 and 2 weeks. Mice were bled 2 weeks after the last immunization. Each serum per group (n=8) was diluted to 1:100 for reaction with gD in ELISA. OD was measured at 405 nm. Values and bars represent individual OD values and the mean, respectively. *Statistically significant at $p<0.05$ by Student's t test compared to each corresponding isotype of gD DNA vaccine alone.

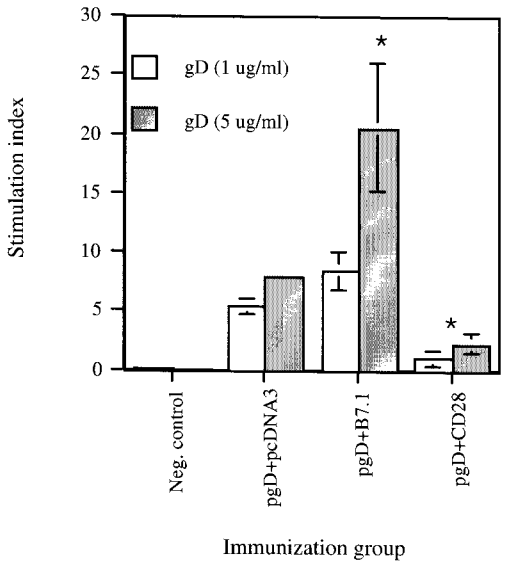

Figure 4. Th cell proliferation levels of splenocytes after *in vitro* gD stimulation. Each group of mice (n=5) was immunized i.m. with gD DNA vaccines (60 μg) plus 40 μg of B7.1 or CD28 molecule cDNAs (40 μg) at 0 and 2 weeks. Two weeks after the last DNA injection, 2-3 mice per group were sacrificed and spleen cells were pooled for the proliferation assay. Splenocytes were stimulated with 1 and 5 μg/ml gD-2 proteins and as a positive control with 5 μg/ml PHA. After three days stimulation, cells were harvested and then cpm was counted. Samples were assayed in triplicate. Values and error bars represent mean and SD, respectively. The PHA control sample showed a stimulation index of 40-50. This represents one of three separate experiments showing similar results. *Statistically significant at $p<0.05$ by Student's t test compared to pgD+pcDNA3.

IgG isotype pattern. gD-specific isotypes were further analyzed to determine if IgG subclasses are driven by coinjection. As shown in Fig. 3, CD28 cDNA coinjection induced a significant increase in IgG1 levels, as compared to gD plasmid vaccine alone. However, coinjection with B7.1 cDNA showed the opposite. These suggest that B7.1 and CD28 are Th1 type and Th2 type-biased, respectively. This shift in IgG production illustrates that a more Th2 type response was induced by CD28 coimmunization in the context of the gD plasmid vaccine.

Th cell proliferative responses. T helper cells play an important role in eliciting both humoral and cellular immune responses via expansion of Ag stimulated B cells and expansion of CD8+ T cells, respectively. As a specific indicator of CD4 activation T cell proliferation was examined. The gD-2 protein (1 and 5 μg/ml) was used for antigen specific stimulation. For a positive control, 5 μg/ml PHA was used as a polyclonal stimulator. As shown in Fig. 4, a low background level of Th cell proliferation was observed in negative plasmid controls. However, gD DNA vaccination induced Th cell proliferation responses much higher than the negative controls. When coinjected with B7.1 plasmid DNA, Th cell proliferation levels were further boosted. However, Th cell proliferation levels were significantly inhibited by coinjection with CD28 cDNAs. This tendency was observed over the two different gD antigen concentrations tested, reflecting that these specific effects are B7.1 or CD28-mediated. A lack of CTL responses

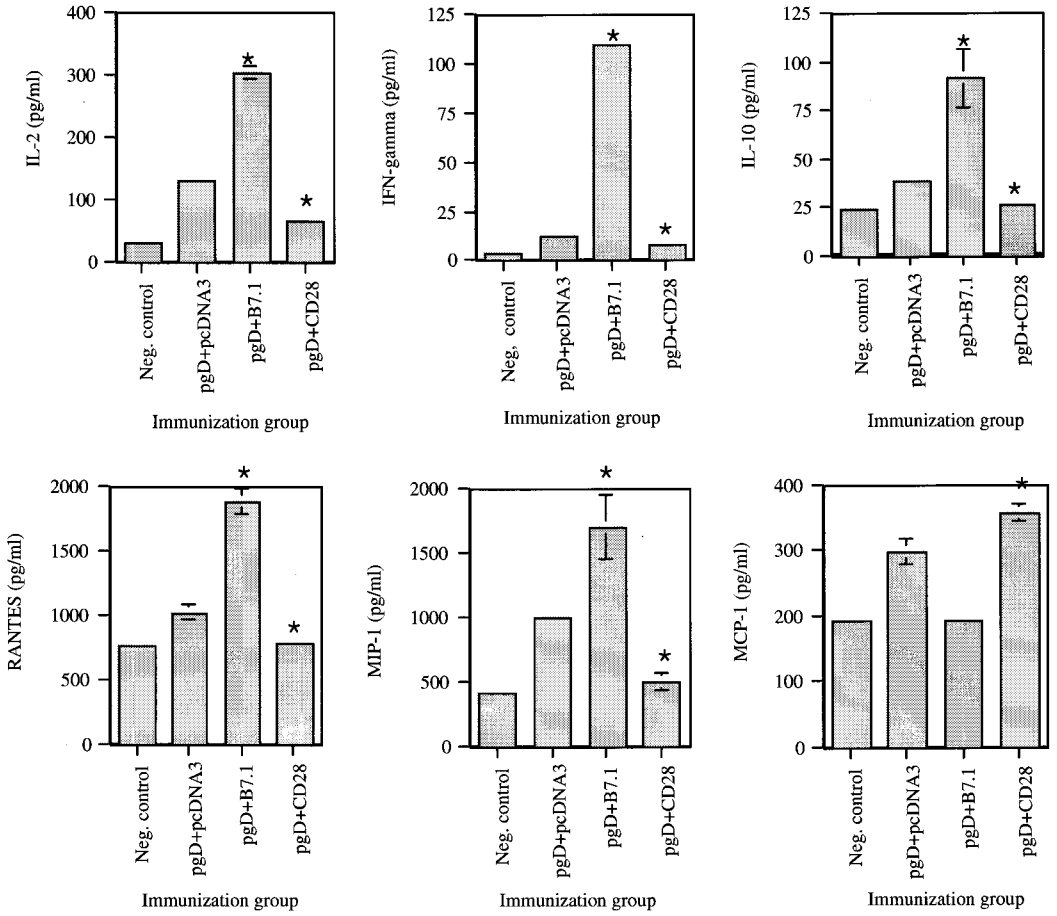

Figure 5. IL-2, IFN-γ, IL-10, RANTES, MIP-1α and MCP-1 production levels of splenocytes after *in vitro* gD stimulation. Each group of mice (n=5) was immunized i.m. with gD DNA vaccines (60 μg) plus 40 μg of B7.1 or CD28 molecule cDNAs at 0 and 2 weeks. Two weeks after the last DNA injection, 2-3 mice per group were sacrificed and spleen cells were pooled. Splenocytes were stimulated with 1 μg of gD proteins/ml for 2 days. Samples were assayed in triplicate. Values and error bars represent mean and SD, respectively. This represents one of three separate experiments showing similar results. *Statistically significant at $p<0.05$ by Student's *t* test compared to pgD+pcDNA3.

against gD in Balb/c mice has been observed previously (23,24). Furthermore, gD plasmid vaccination does not result in CTL responses (17,23,25). Therefore, to evaluate cellular effects in more detail we next examined cytokine and chemokine production profiles.

Levels of Cytokines and Chemokines. Th1 cytokines and Th2 cytokines have been a mainstay in our understanding of the polarization of immune responses. Recently, β-chemokines have also been focused on their ability to polarize immune responses to Th1 or Th2. Th1 immune responses are thought to drive induction of cellular immunity, whereas Th2 immune responses preferentially drive humoral immunity. As shown in Fig. 5, we examined whether gD DNA vaccination with and without B7.1 and CD28 cDNAs modulates Th1 type cytokine and β-chemokine profiles. gD DNA vaccine alone enhanced production of IL-2, IFN-γ, RANTES, MIP-1α and MCP-1 in an antigen-specific manner. In particular, production of IL-2, IFN-γ, RANTES and MIP-1α was enhanced by codelivery with B7.1 DNAs significantly higher than pgD DNA alone. However, MCP-1 production was unaffected by B7.1 coinjection. This suggests that B7.1 enhances Ag- specific Th1 type cellular responses. In the case of CD28 cDNA coinjection, CD28 inhibited production of IL-2, IFN-γ, RANTES and MIP-1α significantly lower than gD DNA vaccine alone. However, MCP-1 production was enhanced, supporting that CD28 inhibits Ag-specific Th1 type cellular responses. These studies support that B7.1 and CD28 costimulate T cells differentially, thus eliciting unique profiles of Th1 type cytokines and β-chemokines

Survival from lethal HSV-2 challenge. It is important that antigen-specific immune modulation influences path-

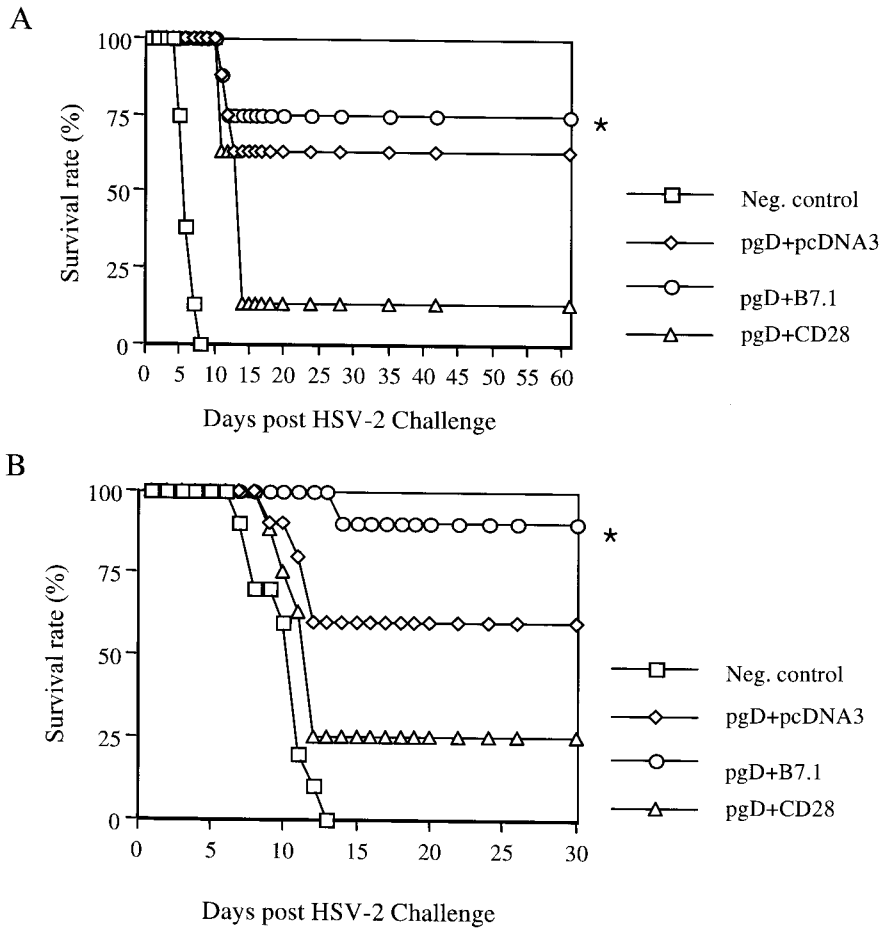

Figure 6. Survival rates of animals immunized with gD DNA vaccines plus costimulatory molecule cDNAs. (A) Each group of mice (n=8) was immunized i.m. with gD DNA vaccines (60 μg) plus B7.1 or CD28 costimulatory molecule cDNAs (40 μg) at 0 and 2 weeks. Three weeks after the last DNA injection, mice were challenged i.vag. with 200 LD_{50} of HSV-2 (7×10^5 pfu). Mice were checked 2 times a week after viral challenge for survival rate studies. (B) Each group of mice (n= 8~10) was immunized once with gD DNA vaccines (10 μg) plus 40 μg of B7.1 or CD28 molecule cDNAs. Five weeks after the DNA immunization, mice were challenged i.vag. with 4 LD_{50} of HSV-2 (1.4×10^4 PFU). Surviving mice were scored for 30 days following viral infection. *Statistically significant at $p<0.05$ by Chi-square test compared to pgD+CD28.

ogen's replication. We analyzed protective efficacy of B7.1 and CD28 coinjection in the murine herpes challenge model. A lethal dose $(LD)_{50}$ of HSV-2 (186) was previously measured to be approximately 3.5×10^3 PFU (18). Eight mice per group were immunized twice with pgD (60 μg per mouse) plus B7.1 or CD28 (40 μg per mouse) and then challenged i.vag. with 200 LD_{50} of HSV-2. As shown in Fig. 6A, all control animals died within 8 days after an intravaginal challenge with 200 LD_{50} of HSV-2. The group immunized with gD DNA vaccine alone had a 63% survival rate after the challenge. In contrast, coinjection with B7.1 cDNA increased the survival rate to 75%, while CD28 coinjection resulted in a dramatic reduction of the survival rate to 13%. To reaffirm the findings above, animals were immunized with 10 μg of pgD vaccines and then challenged with a lower challenge dose of 4 LD_{50} (1.4×10^4 PFU) this time (Fig. 6B). However, the similar findings were also observed. As shown in Fig. 6B, 60% survival of pgD DNA vaccinated animals was noted. When coinjected with B7.1 cDNAs, survival rates were enhanced to 90%, an increase of 30% in survival. However, coinjection with CD28 decreased survival of mice to 25%. As we have previously observed, Th2 bias decreases survival in this model. This further supports the Th2 bias of the CD28 signal. There were no effects on survival in the absence of the specific herpes Ag plasmid, thus supporting that these results do not test the CpG activity of plasmids.

B7.1 reduces morbidity rate after lethal HSV-2 challenge. Mice challenged with 200 LD_{50} of HSV-2 were observed for herpetic lesions for 2 months. Control animals infected with HSV-2 started to show pathological signs, such as lethargy, abnormal gaits, and ruffling fur 2 to 3 days after virus challenge, and they died starting 5 days after infection, and they had all died by 8 days of infection. Surviving mice were then followed for 2 months post challenge to examine pathological symptoms. Herpetic lesions were observed on the epithelial layers of the skin around the vaginal area. As shown in Fig. 7A, immunization with gD DNA vaccine alone resulted in more animals showing herpetic lesions than those which received the gD+B7.1 immunization. For instance, in the case of animals immunized with pgD vaccine alone, the per cent of mice showing herpetic lesions in surviving mice was noted as 100% (5/5),

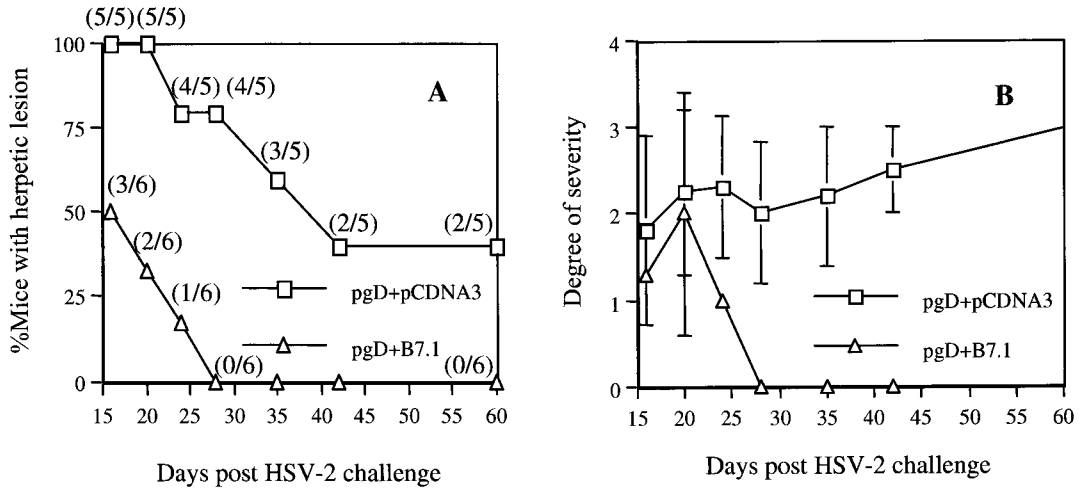

Figure 7. Percent of mice showing herpetic lesions (A) and their severity (B) in mice surviving after HSV-2 infection. Each group of mice (n=8) was immunized and challenged with HSV-2 as shown in Fig. 6A. Mice were checked twice per week after viral challenge to observe the pathological symptoms. (/) indicates the number of mice showing herpetic lesions/the number of mice surviving from HSV-2 challenge. Degrees of severity of herpetic lesions were recorded as tiny lesions (1+), mild (2+), and severe lesions (3+). Values and bars represent the mean of degrees of severity and the SD, respectively.

80% (4/5), 80% (4/5) and 40% (2/5) on days 16, 24, 28 and 61 days post virus challenge, respectively. In the case of pgD+B7.1 immunized animals, however, the per cent of mice showing herpetic lesions in surviving mice was noted as 50% (3/6), 17% (1/6), 0% (0/6) and 0% (0/6) on days 16, 24, 28 and 61 days post virus challenge, respectively. It is notable that coinjection with B7.1 cDNA resulted in complete healing of lesions in surviving animals 28 days post viral challenge. In the case of pgD+ CD28-immunized animals, however, one animal survived, but showed no herpetic lesions (data not shown). We also scored herpetic lesions 1 to 3 based upon their severity (3 being the highest level of severity). As shown in Fig. 7B, coinjection with B7.1 cDNA also induced a significantly lower degree of severity in herpetic lesions, as compared to pgD vaccine alone.

Discussion

Costimulatory molecules play an important role in the initiation and differentiation of CD4+ and CD8+ T cell responses during antigen presentation. Costimulatory molecules, B7.1 and B7.2 expressed on APCs deliver potent costimulatory signals through CD28/CTLA-4 on T cells, resulting in activation of T cells (1,2). These molecules allow APCs and T cells to form a stable T cell and APC conjugate, and they are able to provide signals required for activation of T cells and APCs in context with TCR/Class I or II complex. In various DNA vaccine models, B7.1 or B7.2 co-injected in a DNA form has been observed to have augmented adjuvant efficacy (10-13), suggesting that B7.1 or B7.2 could be used to enhance Ag-specific immune responses *in vivo*. It has, however, been reported that activation of CD28 molecules promotes Th2 type immune responses by activating Th2 promoting transcription factor and increasing IL-4 receptor sensitivity of T cells (5,6). Recently, CD28 costimulation has been reported to induce both type 2 CD4+ and CD8+ T cell polarization (7). This indicates that CD28 could be used to induce antigen-specific Th2 type immune responses *in vivo*.

In this study, there were some effects observed on antibody responses by CD28 coinjection. When coinjected with CD28 cDNAs, there was an enhancement of antibody responses, as compared to gD DNA vaccine alone. However, antibody responses were unaffected by B7.1 coinjection. We further tested IgG isotype production patterns driven by B7.1 and CD28 coinjection. It has been known that IgG1 is a Th2-associated antibody isotype, whereas IgG2a is a Th1-associated isotype antibody (26). We observed that CD28 codelivery induced a significant increase in IgG1 production, which is believed to be mediated by Th2 type cytokine or chemokine induction, suggesting that polarization of gD-specific immune responses towards the Th2 type is achieved by coinjecting plasmid vectors driving expression of CD28. This is also compatible with previous findings that CD28 costimulation is related to differentiation of Th2 type cells (5,6). In contrast, B7.1 coinjection had opposite effects on IgG isotype pattern, as compared to CD28 coinjection, in which IgG2a antibody production was increased while IgG1 produc-

tion was decreased. This is compatible with previous findings that coinjection with B7.1 drives Th1 type responses (12).

As suggested by the isotype pattern, CD28 exhibited distinct effects on overall cytokine induction. In particular, CD28 coinjection inhibited Th cell proliferative responses and production of both Th1 and Th2 type cytokines (IL-2, IFN-γ and IL-10) significantly lower than pgD+pcDNA3, suggesting that CD28 suppresses Ag-specific cellular immune responses *in vivo*. In contrast, B7.1 coinjection enhanced Ag-specific Th cell responses as well as production of both Th1 and Th2 type cytokines (IL-2, IFN-γ and IL-10) significantly higher than pgD+pcDNA3. The data suggest that B7.1 enhances both Th1 and Th2 type cellular immune responses. This property was previously observed by coinjection with GM-CSF and LFA-3 plasmid DNAs (27,28). This is again compatible with previous findings that B7.1 DNA coinjection results in enhanced cellular responses in a gD DNA vaccine model (12).

Beta chemokines, RANTES, MCP-1, and MIP-1α chemoattact particularly for monocytic phagocytes and are also known to activate T cells, basophils, eosinophils, and mononuclear phagocytes (29,30). In particular, RANTES chemoattracts unstimulated CD4+/CD45RO+ memory T cells and stimulated CD4+ and CD8+ T cells (31-34). MIP-1α and MCP-1 also stimulate Th1 or Th2 type cytokine production from T cells (34,35). Evidence suggests that chemokines could also be divided into Th1 and Th2 phenotypes (36,37) in a similar manner to cytokines. In support of this theory, we reported that coinjection with a Th1 type cytokine, IL-12 enhances production of RANTES and MIP-1α, but suppressed MCP-1 production (17). In contrast, subunit vaccine was a strong Th2 inducer and enhanced production of MCP-1, but suppressed production of RANTES and MIP-1α (20). This report combined with the present study suggested that MCP-1 could be considered as an inducer of a Th2 type response. Further support for this idea is that Th1 cells which mediate ocular inflammatory disease by HSV infection can be ameliorated by injection with anti-MIP-1α but not anti-MCP-1 (38), again indicating that MIP-1α and MCP-1 might have conflicting roles in the Th1 and Th2 type cell-mediated immune responses, respectively. In this study, CD28 inhibited production of RANTES and MIP-1α, but enhanced production of MCP-1 as compared to pgD+pcDNA3, supporting the Th2 bias of antibody responses by CD28. This is also in line with the finding in which coinjection with MCP-1 drives Th2-biased immune responses (35,39). It seems likely that MCP-1 activates progression to Th2 type immune responses, resulting in loss of protection against HSV-2. This is compatible with the previous finding that coinjection with MCP-1 cDNA makes animals more susceptible for HSV-2 challenge (40).

As suggested by enhancement of cellular responses by B7.1, B7.1 increased protection from mortality and morbidity resulting from a lethal dose of HSV-2. However, CD28, an inducer of Th2 type immune responses showed increased susceptibility to HSV-2 challenge. We previously observed in DNA vaccine studies that antibody responses are not correlated with enhanced protection from HSV challenge by codelivery with pgD+IL-12 DNAs (17). This suggests that the quality of help provided by B7.1 particularly relates to cellular responses. It is also likely that B7.1-induced IFN-γ is likely responsible for the anti-HSV-2 activity of these molecules *in vivo*. Direct anti-HSV effects of IFN-γ were previously reported in HSV infection (41) as well as hepatitis B virus and lymphocytic choriomeningitis virus infection models (42,43), supporting that IFN-γ is critical for anti-HSV control. However, in addition to IFN-γ Th1 or Th2 polarization might be modulated by the costimulatory molecules.

In conclusion, the data presented here support that the costimulatory molecule CD28 drives immune responses in a Th2 phenotype. Recent studies of the mechanism of DNA vaccination allow us to speculate on this activity. Plasmid vaccines transfect local cells at the site of injection. In the case of i.m. injection, both muscle cells as well as resident APCs are transfected (44,45). These transfected APCs migrate to the regional lymph node for immune stimulation. There is little evidence to support transfection of lymphocytes including B cells. It has been well established that mixing plasmids allows for coexpression of encoded sequences largely within the same cells. Accordingly, there are two major places that CD28 can play a role in this model, the peripheral tissue site (muscle) or the regional lymph node expressed on APCs. It is unlikely that CD28 can stimulate T cells directly in the periphery (muscle) as neither the IL-2 or B7 signal would be provided at the necessary time. However, CD28 should be able to activate APCs in the muscle in the area of the gD antigen as plasmid vaccination is depo delivery. These APCs would be expected to migrate to the regional lymph nodes and these antigen armed activated APCs would be expected to be effector expanders of the developing host immune response.

In sum, the data presented here suggest that B7.1 and CD28 have distinct costimulatory pathways for inducing antigen-specific humoral or cellular responses which result in different types of protective immunity against HSV-2 infection *in vivo*.

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References

1. Carreno BM, Collins M: The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Ann Rev Immunol 20;29-53, 2002
2. Sharpe AH, Freeman GJ: The B7-CD28 superfamily. Nat Rev Immunol 2;116-126, 2002
3. Lanier LL, O'Fallon S, Somoza C, Phillips JH, Linsley PS, Okumura K: CD80 (B7) and CD86 (B70) provide similar costimulatory signals for T-cell proliferation, cytokine production, and generation of CTL. J Immunol 154;97-105, 1995
4. June C, Bluestone JA, Nadler LM, Thompson CB: The B7 and CD28 receptor families. Immunol Today 15;321-333, 1994
5. Kubo M, Yamashita M, Abe R, Tada T, Okumura K, Ransom JT, Nakayama T: CD28 costimulation accelerates IL-4 receptor sensitivity and IL-4-mediated Th2 differentiation. J Immunol 163;2432-2442, 1999
6. Rodriguez-Palmero M, Hara T, Thumbs A, Hunig T: Triggering of T cell proliferation through CD28 induces GATA-3 and promotes T helper type 2 differentiation *in vitro* and *in vivo*. Eur J Immunol 29;3914-3924, 1999
7. Bian Y, Hiraoka S, Tomura M, Zhou XY, Yashiro-Ohtani Y, Mori Y, Shimizu J, Ono S, Dunussi-Joannopoulos K, Wolf S, Fujiwara H: The capacity of the natural ligands for CD28 to drive IL-4 expression in naive and antigen-primed CD4+ and CD8+ T cells. Int Immunol 17;73-83, 2005
8. Orabona C, Grohmann U, Belladonna ML, Fallarino F, Vacca C, Bianchi R, Bozza S, Volpi C, Salomon BL, Fioretti MC, Romani L, Puccetti P: CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. Nat Immunol 5;1134-1142, 2004
9. Miyahira Y, Katae M, Kobayashi S, Takeuchi T, Fukuchi Y, Abe R, Okumura K, Yagita H, Aoki T: Critical contribution of CD28-CD80/CD86 costimulatory pathway to protection from Trypanosoma cruzi infection. Infect Immun 71;3131-3137, 2003
10. Iwasaki A, Stiernholm BJ, Chan AK, Berstein NL, Barber BH: Enhanced CTL responses mediated by plasmid DNA immunogens encoding costimulatory molecules and cytokines. J Immunol 158;4591-4601, 1997
11. Kim JJ, Bagarazzi ML, Trivedi N, Hu Y, Chattergoon MA, Dang K, Mahalingam S, Agadjanyan MG, Boyer JD, Wang B, Weiner BD: Engineering of *in vivo* immune responses to DNA immunization via co-delivery of costimulatory molecule genes. Nat Biotech 15;641-645, 1997
12. Flo J, Tisminetzky S, Baralle F: Modulation of the immune response to DNA vaccine by co-delivery of costimulatory molecules. Immunol 100;259-267, 2000
13. Maue AC, Waters WR, Palmer MV, Whipple DL, Minion FC, Brown WC, Estes DM: CD80 and CD86, but not CD154, augment DNA vaccine-induced protection in experimental bovine tuberculosis. Vaccine 23;769-779, 2004
14. Nahmias AJ, Dannenbarger J, Wickliffe C, Muther J: Clinical aspects of infection with herpes simplex virus 1 and 2. Elsevier, New York, 1980
15. Kuklin NA, Daheshia M, Chun S, Rouse BT: Role of mucosal immunity in herpes simplex virus infection. J Immunol 160;5998-6003, 1998
16. Yu Z, Manickan E, Rouse BT: Role of interferon-gamma in immunity to herpes simplex virus. J Leuko Biol 60;528-532, 1996
17. Sin JI, Kim JJ, Arnold RL, Shroff KE, McCallus D, Pachuk C, McElhiney SP, Wolf MW, Pompa-de Bruin SJ, Higgins TJ, Ciccarelli RB, Weiner DB: Interleukin-12 gene as a DNA vaccine adjuvant in a herpes mouse model: IL-12 enhances Th1 type CD4+ T cell mediated protective immunity against HSV-2 challenge. J Immunol 162;2912-2921, 1999
18. Sin JI, Kim JJ, Boyer JD, Higgins TJ, Ciccarelli RB, Weiner DB: *In vivo* modulation of vaccine-induced immune responses toward a Th1 phenotype increases potency and vaccine effectiveness in a herpes simplex virus type 2 mouse model. J Virol 73;501-509, 1999
19. Sin JI, Bagarazzi M, Patchuk C, Weiner DB: DNA priming-protein boosting enhances both Ag-specific antibody and Th1 type cellular immune responses in a murine herpes simplex virus-2 gD vaccine model. DNA Cell Biol 18;771-779, 1999
20. Sin JI, Ayyavoo V, Boyer J, Kim J, Ciccarelli RB, Weiner DB: Protective immune correlates can segregate by vaccine type in a murine herpes model system. Int Immunol 11; 1763-1773, 1999
21. McDermott MR, Smiley JR, Leslie P, Brais J, Rudzroga HE, Bienenstock J: Immunity in the female genital tract after intravaginal vaccination of mice with an attenuated strain of herpes simlex virus type 2. J Virol 51;747-753, 1984
22. Milligan GN, Bernstein DI: Generation of humoral immune responses against herpes simplex virus type 2 in the murine female genital tract. Virol 206;234-241, 1995
23. Ghiasi H, Cai S, Slanina S, Nesburn AB, Wechsler SL: Vaccination of mice with herpes simplex virus type 1 glycoprotein D DNA produces low levels of protection against lethal HSV-1 challenge. Antiviral Res 28;147-157, 1995
24. Martin S, Moss B, Berman PW, Laskey LA, Rouse BT: Mechanisms of antiviral immunity induced by a vaccine virus recombinant expressing herpes simplex virus type 1 glycoprotein D: cytotoxic T-cells. J Virol 61;726-734, 1987
25. Cruz PE, Khalil PL, Dryden TD, Chiou HC, Fink PS, Berberich SJ, Bigley NJ: A novel immunization method to induce cytotoxic T-lymphocyte responses (CTL) against plasmid-encoded herpes simplex virus type-1 glycoprotein D. Vaccine 17;1091-1099, 1999
26. Snapper CM, Paul WE: Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 236;944-947, 1987
27. Sin JI, Kim JJ, Dang K, Lee D, Patchuk C, Satishchandran C, Weiner DB: LFA-3 plasmid DNA enhances Ag-specific humoral and cellular mediated protective immunity against herpes simplex virus-2 *in vivo*: Involvement of CD4+ T cells in protection. Cell Immunol 203;19-28, 2000
28. Sin JI, Kim JJ, Ugen KE, Ciccarelli RB, Higgins TJ, Weiner DB: Enhancement of protective humoral (Th2) and cell-mediated (Th1) immune responses against herpes simplex virus-2 through co-delivery of granulocyte macrophage-colony stimulating factor expression cassettes. Eur J Immunol 28;3530-3540, 1998
29. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K: Properties of the novel proinflammatory supergene "intercrine" cytokine family. Ann Rev Immunol 9;617-648, 1991
30. Schall TJ: Biology of the RANTES/SIS cytokine family. Cytokine 3;165-183, 1991
31. Schall TJ, Bacon K, Toy KJ, Goeddel DV: Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 347;669-671, 1990

32. Murphy WJ, Taub DD, Anver M, Conlon K, Oppenheim JJ, Kelvin DJ, Longo DL: Human RANTES induces the migration of human T lymphocytes into the peripheral tissues of mice with severe combined immune deficiency. Eur J Immunol 24;1823-1827, 1994
33. Meurer R, Van Riper G, Feeney W, Cunningham P, Hora Jr. D, Springer MS, MacIntyre DE, Rosen H: Formation of eosinophilic and moncytic intradermal inflammatory sites in the dog by injection of human RANTES but not human monocyte chemoattractant protein 1, human macrophage inflammatory protein 1 alpha, or human interleukin 8. J Exp Med 178;1913-1921, 1993
34. Taub DD, Turcovski-Corrales SM, Key ML, Longo DL, Murphy WJ: Chemokines and T lymphocyte activation: I. Beta chemokines costimulate human T lymphocyte activation *in vitro*. J Immunol 156;2095-2103, 1996
35. Karpus WJ, Kennedy KJ, Kunkel SL, Lukacs NW: Monocyte chemotactic protein 1 regulates oral tolerance induction by inhibition of T helper cell 1-related cytokines. J Exp Med 187;733-741, 1998
36. Kim JJ, Nottingham LK, Sin JI, Tsai A, Morrison L, Dang K, Hu Y, Kazahaya K, Bennett M, Dentchev T, Wilson DM, Chalian AA, Boyer JD, Agadjanyan MG, Weiner DB: CD8 positive T-cells influence antigen-specific immune responses through the expression of chemokines. J Clin Invest 102; 1112-1124, 1998
37. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A: Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 187; 875-883, 1998
38. Tumpey TM, Cheng H, Yan XT, Oakes JE, Lausch RN: Chemokine synthesis in the HSV-1-infected cornea and its suppression by interleukin-10. J Leuko Biol 63;486-492, 1998
39. Lukacs NW, Chensue SW, Karpus WJ, Lincoln P, Keefer C, Strieter RM, Kunkel SL: C-C chemokines differentially alter interleukin-4 production from lymphocytes. Am J Pathol 150;1861-1868, 1997
40. Sin JI, Kim JJ, Pachuk C, Satishchanran C, Weiner DB: DNA vaccines encoding interleukin-8 and RANTES enhance antigen-specific Th-1 type CD4+ T cell-mediated protecetive immunity against herpes simplex virus-2 *in vivo*. J Virol 74; 11173-11180, 2000
41. Ho M: Interferon as an agent against herpes simplex virus. J Investig Dermatol 95;S158-S160, 1990
42. Guidotti LG, Rochford R, Chung J, Shapiro M, Purcell R, Chisari FV: Viral clearance without destruction of infected cells during acute HBV. Science 284;825-829, 1999
43. Guidotti LG, Borrow P, Brown A, McClary H, Koch R, Chisari FV: Noncytopathic clearance of lymphocyte choriomeningitis virus from the hepatocyte. J Exp Med 189;1555-1564, 1999
44. Chattergoon MA, Robinson TM, Boyer JD, Weiner DB: Specific immune induction following DNA-based immunization through *in vivo* transfection and activation of macrophages. J Immunol 160;5707-5718, 1998
45. Sin JI, Kim J, Chattergoon M, Ayyavoo V, McCallus D, Ugen KE, Boyer JD, Weiner DB: Engineering of DNA vaccines using molecular adjuvant plasmids. Dev Biol (Basel) 104; 187-198, 2000