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IL-12 and IL-18 act in synergy to clear vaccinia virus infection: involvement of innate and adaptive components of the immune system

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Development of a protective host response against intracellular pathogens requires innate and cell-mediated immune responses, with cytokines playing an important role in host defences. Different studies in mice have shown that IL-12 can promote protective immunity to a variety of viruses but, during virus infection, little is known about the *in vivo* function of IL-18 alone or in combination with IL-12. Using recombinant vaccinia viruses (rVVs) expressing IL-12 and IL-18, the antiviral role of both cytokines in mice has been analysed. The specific anti-VV immune response elicited and the persistence of the virus in target tissues were compared in BALB/c mice inoculated with rVVs expressing IL-12 and IL-18 either singly or in combination. Delivery of IL-12 and IL-18 by rVVs in mice induced a significant enhancement in virus clearance from ovaries and spleen, greater than that expected from the sum of action of both cytokines. Virus clearance involved NK and T cells, as demonstrated in mice depleted of NK cells and in immunodeficient SCID animals. Th1 parameters ($CD8^+$ T cell response and IgG2a : IgG1 ratios) were increased in mice inoculated with rVVs expressing both IL-12 and IL-18 as compared to those animals receiving a single cytokine. These findings indicate that when IL-12 and IL-18 are delivered by rVVs, different mechanisms involving both the innate and specific arms of the immune system act as mediators in the synergistic action of IL-12 and IL-18, leading to VV clearance. These results are of interest for the design of prophylactic as well as therapeutic VV-based strategies.

INTRODUCTION

Development of an effective immune response to foreign pathogens is achieved by a complex interaction of a variety of immune-competent cells, such as T cells, B cells, NK cells and macrophages. Virus infections generally induce immune responses that lead to long lasting immune memory, in which $CD8^+$ T cells are key mediators of protective immunity against both non-cytopathic and cytopathic viruses (Zinkernagel, 1996; Ramsay *et al.*, 1993). Cytokines secreted by immune cells play an important role in host defences against virus infections. IL-12 induction has been demonstrated during infections with RNA and DNA viruses (Coutelier *et al.*, 1995; Kanangat *et al.*, 1996; Orange & Biron, 1996) and different studies in mice have shown that this cytokine can promote protective immunity to a variety of viruses (Ozmen *et al.*, 1995; Gazzinelli *et al.*, 1994; Orange *et al.*, 1995; Cavanaugh *et al.*, 1997). IL-18, a cytokine identified recently, is secreted by antigen-presenting cells (APCs) and, during infection, induces IFN-γ production primarily in primed T cells and NK cells in synergy with IL-12 (Dinarello *et al.*, 1998). IL-18 shares biochemical properties with IL-1 and functional similarities with IL-12; thus, IL-18 is synthesized as an inactive precursor protein (21 kDa) that is cleaved by caspase-1 to produce the biologically active protein of 19 kDa (Okamura *et al.*, 1995). The significance of IL-18 in the development of antiviral immune responses has been provided with some viruses. It has been described that after infection of human macrophages with influenza virus, the cells produce IL-18, which acts synergistically with IFN-α and enhances IFN-γ synthesis (Sareneva *et al.*, 1998), and IL-18 treatment induces protection against herpes simplex virus infection by both IFN-γ-dependent and -independent pathways (Fujioka *et al.*, 1999). During murine cytomegalovirus infection, it was demonstrated that IL-18 induced compartmental IFN-γ responses during virus infection (Pien *et al.*, 2000). On the contrary, during respiratory syncytial virus infection in mice, IFN-γ signalling is required for a Th1 protective response, but IL-12 and IL-18 are not involved (Boelen *et al.*, 2002). Moreover, during vesicular stomatitis virus infection of the CNS, no IL-18 role in host protection was found (Hodges *et al.*, 2001).

Several studies have analysed the role of different cytokines during infection with vaccinia virus (VV): some increased virulence, as with IL-4, whereas others attenuated the virus,

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as with IFN-γ or TNF-α (Ramshaw *et al.*, 1997). We have described previously that in mice inoculated with recombinant VV (rVV) expressing IL-12, this cytokine attenuates the virus by inducing high levels of IFN-γ and incrementing both the antiviral CTL response as well as the specific Th1 : Th2 ratio (Gherardi *et al.*, 1999). Moreover, another report (van den Broek *et al.*, 2000) also pointed out the importance of this cytokine in VV infections and suggested that IL-12 is more important than IFN-γ against VV. IL-18 displays similar effects as IL-12; thus, it is tempting to speculate that IL-18 might have a protective role against VV infection. In this sense, it has been described that administration of recombinant IL-18 to mice infected with VV induces an antiviral effect mediated by different mechanisms that include NK and CTL cells (Tanaka-Kataoka *et al.*, 1999). Interestingly, the potential importance of this cytokine against poxvirus infections is suggested by the finding of different poxvirus genes related to IL-18 that contribute to virus evasion of the host immune system (Xiang & Moss, 1999; Bowie *et al.*, 2000; Symons *et al.*, 2002).

While synergy between IL-12 and IL-18 has been observed in different model systems (Fukao *et al.*, 2000; Yamanaka *et al.*, 1999; Hofstra *et al.*, 1998), there is little information of this effect in the context of a virus infection. The aim of this study was to analyse the effect of IL-18, either alone or in combination with IL-12, on VV infection in the murine model. We have defined the importance of both cytokines delivered from rVV in virus clearance and in the generation of non-specific and specific anti-VV immune responses.

METHODS

Viruses and cells. VV recombinants were derived from the Western Reserve (WR) strain. Both rVVHA$^-$ and rVVIL-12, described previously (Gherardi *et al.*, 1999), express the luciferase gene in the thymidine kinase (TK) locus (tk$^-$) under the control of the early 7.5 promoter and the haemagglutinin gene (HA), which was disrupted (HA$^-$) or interrupted by a cassette encoding the p35 and p40 subunits of murine IL-12, respectively (Fig. 1A). The recombinant virus rVVIL-18 was generated for this study and expresses the luciferase gene in the TK locus and the IL-18 gene in the HA locus. IL-18 expression is under the control of a VV synthetic early/late promoter (Fig. 1A, e/l). Thus, all rVVs used are HA and TK negative.

Viruses were grown in HeLa cells, titrated in African green monkey kidney BSC40 cells and purified through a sucrose cushion, as described previously (Dallo & Esteban, 1987). The murine macrophage cell line J774 was grown in complete RPMI medium supplemented with 10 % FCS.

Immunizations of mice and serum sample collection. BALB/c mice (*H-2*^{d}), SCID BALB/c mice or C57BL/6 (*H-2*^{b}) mice, 6 to 8 weeks old, were inoculated intraperitoneally with different doses of the different rVVs in 200 µl sterile PBS. Blood was obtained at different times post-infection (p.i.) from the retroorbital plexus. Serum was then isolated and stored at −20 °C.

Measurement of luciferase activity in mouse tissues. Replication of rVVs in different mouse tissues was followed by a highly sensitive luciferase assay that correlates with virus titres *in vivo* (Rodríguez *et al.*, 1988). Briefly, organs from euthanized animals were homogenized in luciferase extraction buffer (300 µl per spleen and 100 µl per ovary) (Promega) and cleared supernatants were obtained. Luciferase activity was measured according to manufacturer's instructions in a Lumat LB 9501 Berthold luminometer (Berthold) and was expressed as relative luciferase units (RLU) mg^{-1} protein. Protein content in tissue extracts was measured with the Bicinchoninic Acid Protein Assay kit (Pierce).

Evaluation of cytokine levels by ELISA. Cytokine levels were evaluated in serum and in clarified spleen homogenates, performed in PBS containing protease inhibitors (Complete, Roche). ELISA determinations used the appropriate combination of antibodies and followed the instructions of the manufacturer (Pharmingen).

Evaluation of CD8$^+$ T cells by the ELISPOT assay. The ELISPOT assay to detect antigen-specific CD8$^+$ T cells was performed as described previously (Ramírez *et al.*, 2000). Antibodies used were anti-mouse IFN-γ mAb R4-6A2 and biotinylated anti-mouse IFN-γ mAb XMG1.2 (Pharmingen). P815 cells, a mastocytome cell line that expresses only MHC class I molecules (Miyahira *et al.*, 1995) were used as APCs. The number of specific CD8$^+$ T cell anti-VV antigens was evaluated by infecting P815 cells at an m.o.i. of 5 p.f.u. per cell. At 4·5 h p.i., cells were washed and treated with mitomycin C (30 µg ml^{-1}) (Sigma). Development was performed with peroxidase-labelled avidin (Sigma) by adding 3,3′-diaminobenzidine tetrahydrochloride (Sigma). Spots were counted with the aid of a stereomicroscope.

Antibody measurements by ELISA. ELISA was used to determine the presence of antibodies against VV antigens in serum samples. VV antigens (1 µg ml^{-1}) used to coat 96-well flat-bottomed plates consisted of envelope proteins extracted from purified virions, as described previously (Rodríguez *et al.*, 1997). Peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology) antibodies were diluted 1 : 1500 and 1 : 2000, respectively, in blocking buffer and incubated for 1 h at 37 °C. Finally, absorbance values were measured at 492 nm on a Labsystems Multiskan Plus plate reader.

NK cell depletion. NK cells were depleted from 6- to 8-week-old C57BL/6 mice 1 day before virus inoculation. Mice were inoculated intraperitoneally with 100 µg purified mAb PK136 in PBS (1 mg ml^{-1}), which is specific for the pan-NK surface marker NK-1.1 (a generous gift from Werner Held, Ludwig Institute, Lausanne, Switzerland), or with normal serum. NK cell depletion was measured by FACS analysis using the pan-NK DX5 antibody (Pharmingen) in splenocyte suspensions. Levels of depletion were consistently between 60 and 80 % (data not shown).

Statistical analyses. Statistical analyses were performed by applying the unpaired Student *t*-test. Significant differences were defined with $P<0{\cdot}01$ and $P<0{\cdot}05$.

RESULTS

Expression of IL-18 from rVV decreases virus replication in target mouse tissues

To study the *in vivo* antiviral effect of IL-18 against a poxvirus infection, we generated for this study rVVIL-18, which expresses the complete gene of IL-18 and the luciferase reporter gene. Fig. 1(A) shows the recombinant viruses employed in this study. The complete inactive (21 kDa) and the processed (19 kDa) forms of IL-18 were detected by Western blot analysis of cell extracts (Fig. 1B,

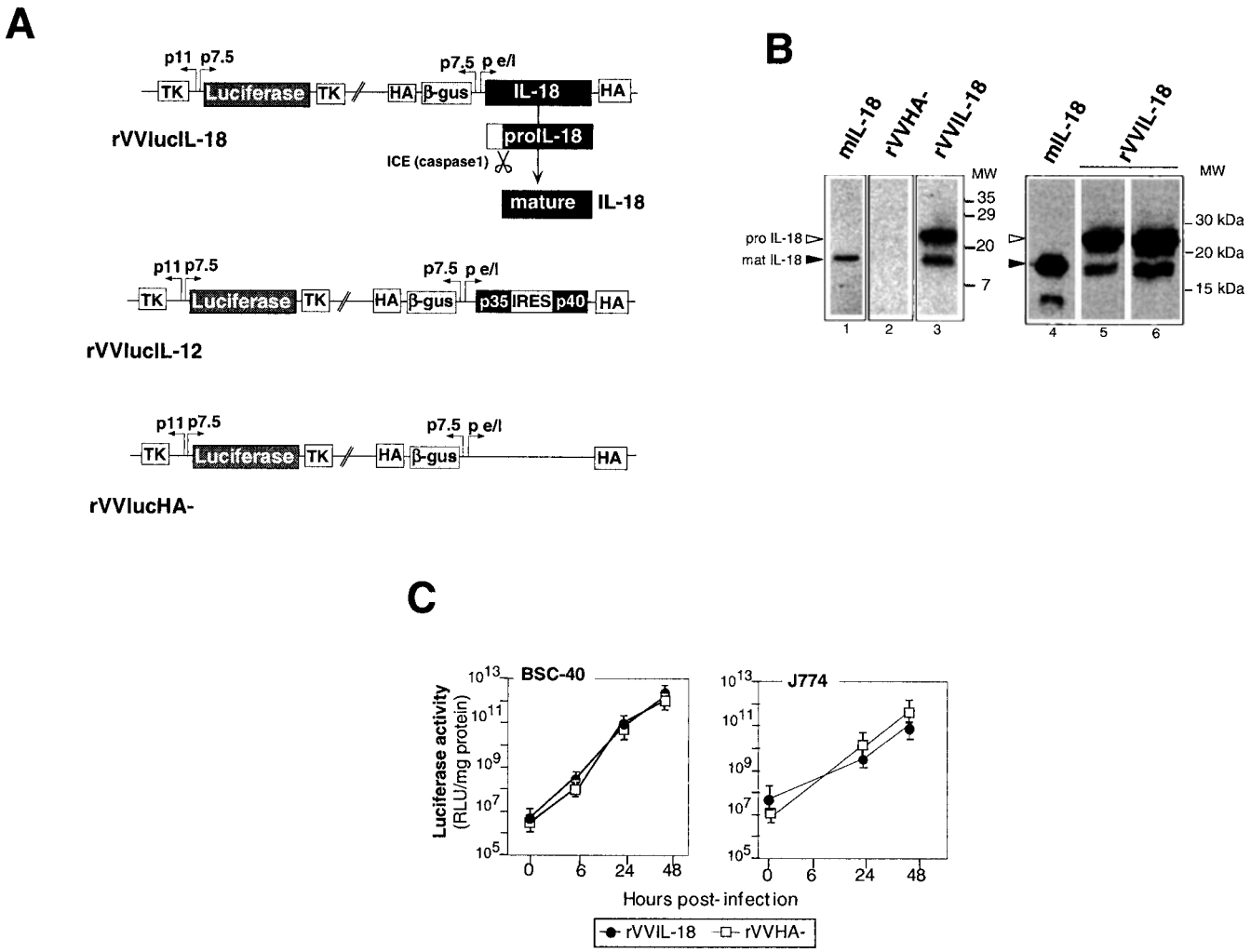

Fig. 1. Characterization of rVVIL-18. (A) The genes encoding the IL-18 and IL-12 proteins were inserted into the HA locus and the gene encoding the luciferase protein was inserted into the TK locus of the VV genome. (B) Western blot analysis of IL-18 expression from J774 cells. Monolayers of J774 cells were infected (10 p.f.u. per cell) with the rVVs indicated and cell extracts at 24 h p.i. were fractionated by 14% SDS-PAGE under reducing conditions, transferred to a nitrocellulose filter and reacted with a rat anti-IL-18 mAb (mAb 422, RD Systems). Antibody reactivity was detected by immunoperoxidase staining using standard procedures. Lanes: 1 and 4, standard mouse recombinant IL-18; 2, cell extract from rVVHA$^-$-infected cells; 3 and 5, cell extracts from rVVIL-18-infected cells; 6, supernatant from rVVIL-18-infected cells. (C) Kinetics of rVV-driven luciferase gene expression in BSC40 (left panel) or J774 (right panel) cell lines infected with an m.o.i. of 0·01 p.f.u. per cell of rVVHA$^-$ or rVVIL-18. At each time-point, cells were scraped and lysed and luciferase levels in clarified supernatants were then measured. Average values $\pm$SD from duplicate cultures are shown.

lanes 3 and 5) and also in cell supernatants (Fig. 1B, lane 6) derived from the murine macrophage cell line J774 infected with rVVIL-18. However, no IL-18 expression was detected in cells infected with rVVHA$^-$ (Fig. 1B, lane 2). The size of the processed protein was the same as that of purified IL-18 (Fig. 1B, lanes 1 and 4). Fig. 1(C) shows the levels of luciferase with time of infection with rVVIL-18 or rVVHA$^-$, being similar in both BSC40 and J774 cell lines. Thus, expression of the cytokine has no effect on virus growth in cultured cells. Next, we analysed if IL-18 delivery from rVV has any effect on virus replication *in vivo*. To do this, BALB/c mice were inoculated intraperitoneally with 10^8 p.f.u. rVVIL-18 or rVVHA$^-$ and the luciferase activity present in the ovaries was measured. At 1–3 days p.i., similar levels of luciferase were found in both groups of animals, whereas at 5 days p.i., luciferase levels in the ovaries of mice inoculated with rVVIL-18 were 25-fold lower than in the control group ($P=0{\cdot}03$) (Fig. 2A). At 7 and 8 days p.i., luciferase levels in mice inoculated with rVVIL-18 were 3- and 1·5-fold lower, respectively, with respect to the control group (data not shown). Importantly, we found a better health status in animals inoculated with rVVIL-18 compared with animals

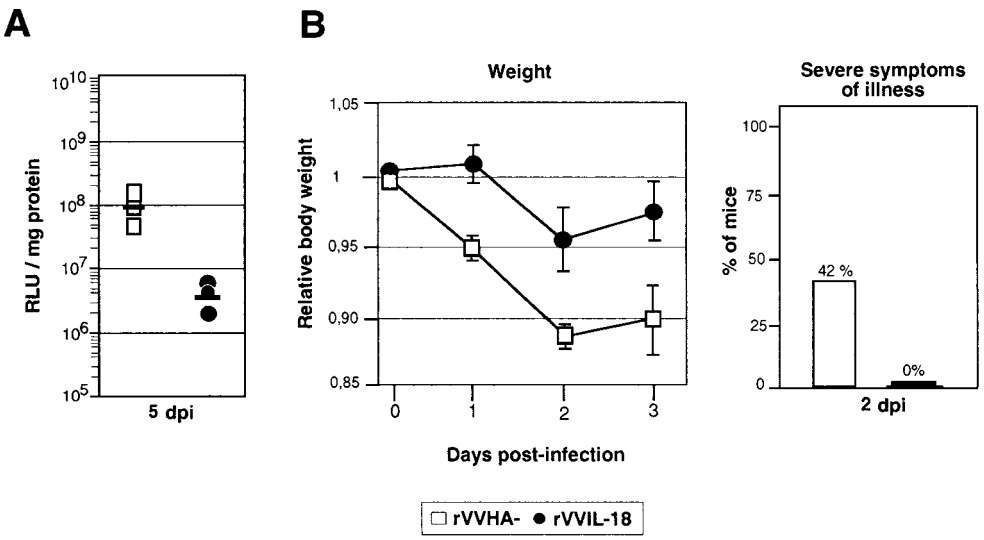

Fig. 2. *In vivo* characterization of rVV expressing murine IL-18. (A) Groups of BALB/c mice were inoculated intraperitoneally with 1×10^8 p.f.u. per mouse of the virus indicated. Luciferase activity (expressed as RLU mg^{-1} protein) detected in ovary tissue homogenates at 5 days p.i. is depicted. Each symbol corresponds to an individual mouse and the thick bar indicates the average value. (B) Groups of 12 mice were inoculated as in (A) and weighed from 0 to 3 days p.i. Changes in body weight are expressed as the relative body weight at each time-point in reference to the weight observed at day 0. Values are the mean ± SD (left panel). At 2 days p.i., mice with strong symptoms of illness (reduced mobility, ruffled fur, arched backs and eye necropsy) were counted (right panel).

inoculated with rVVHA$^-$, as shown by measuring loss of body weight (Fig. 2B, left panel) and characteristic signs of illness (reduced mobility, ruffled fur, arched backs and eye necropsy) (Fig. 2B, right panel).

These results demonstrated that in cultured cells, IL-18 delivered by rVV has no effect on virus replication. In contrast, however, IL-18 in mice decreases VV replication with time and significantly reduces the signs of the virus infection process.

IL-12 and IL-18 delivered from rVV promote a synergistic attenuation of the virus

Next, we analysed if simultaneous expression of IL-12 and IL-18 from different rVVs could enhance the antiviral efficacy of a single cytokine. Four groups of BALB/c mice were inoculated intraperitoneally with different combinations of rVVs; all animals received a total dose of 6×10^7 p.f.u. of virus expressing the luciferase reporter gene (Fig. 3). The dose of 1×10^7 p.f.u. rVVIL-12 was chosen to diminish IL-12-mediated anti-VV effects observed previously (Gherardi *et al.*, 1999) and, thus, allowed us to monitor its potential benefit during the expression of both cytokines. At different days p.i., levels of luciferase activity were measured in the ovaries and spleen (Fig. 3). In ovaries from mice co-inoculated with the rVVs expressing IL-12 and IL-18 (group IV), there is, from days 2 to 7 p.i., a decrease in virus replication compared to the groups inoculated with rVVs expressing either IL-12 (group III) or IL-18 (group II). Higher differences between groups were observed at 3 and 4 days p.i. Luciferase activities in groups III and IV were significantly different relative to control-infected mice ($P<0{\cdot}01$) (rVVHA$^-$) from 2 days p.i. up to 7 days p.i., whereas rVVIL-18-infected mice showed a 6- and 9-fold drop ($P<0{\cdot}01$) in luciferase activity at 4 and 7 days p.i., respectively, compared with rVVHA$^-$-infected mice. We observed in ovaries at both 3 and 4 days p.i. that co-delivery of rVVIL-12 and rVVIL-18 induced a synergistic activity. Thus, compared to group I (control group) and at 3 days p.i., mice from group IV (rVVIL-12 + rVVIL-18) showed 300-fold lower levels of luciferase, while mice inoculated with either rVVIL-18 or rVVIL-12 had 3 and 15 times less luciferase activity, respectively. At this time-point, differences between groups IV and III were significantly different ($P<0{\cdot}05$). Moreover, at 4 days p.i., luciferase activity in group IV was nearly 250-fold lower than that in the control group, whereas in groups III and II, the drop was 50- and 6-fold, respectively. When luciferase activity was measured in the spleen, greater differences between the groups were observed at 2 days p.i. This is probably due to the faster kinetics of VV clearance in this organ than in ovaries. In the spleen, no significant differences in luciferase levels were found between animals given rVVIL-18 (group II) with respect to those given the control, rVVHA$^-$ (group I). However, data between groups III and IV with respect to group I were statistically significant ($P<0{\cdot}01$) at 2 and 3 days p.i. At 2 days p.i., luciferase expression in the spleen of

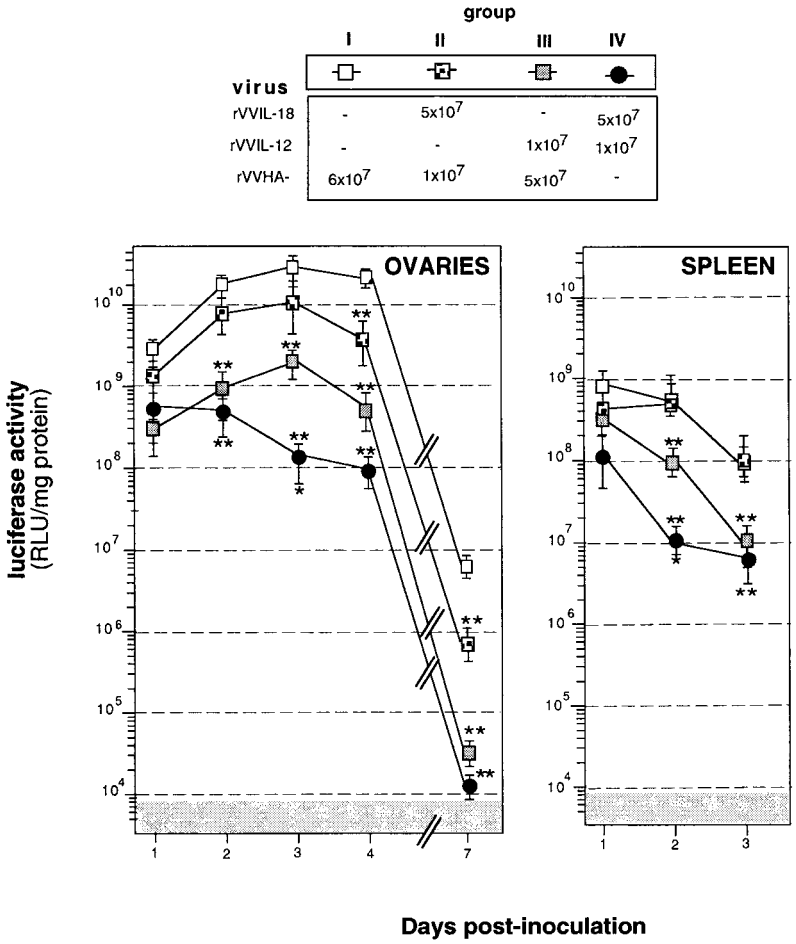

Fig. 3. Expression of IL-12 and IL-18 from rVVs produced a synergistic protective effect against virus infection. Mice were inoculated intraperitoneally with the doses and combination of viruses depicted in the upper part of the figure to a final dose of rVV expressing luciferase of 6×10^7 p.f.u. At the times indicated, ovaries and spleen from four mice were processed and luciferase activity was measured, as described in Methods, and related to protein content. Each point represents mean values from four mice $\pm$SD. Significant differences relative to the control group (group I) are denoted as ** ($P<0{\cdot}01$); significant differences between groups III and IV are denoted as * ($P<0{\cdot}05$). A representative experiment of three is shown.

animals from group IV was 50-fold lower than that in control mice, whereas in mice from group III these values were 5-fold lower (10-fold lower levels of luciferase in group IV than in group III, $P<0{\cdot}05$). Thus, these data showed that at three time-points p.i., namely days 3 and 4 in the ovaries and day 2 in the spleen, co-delivery of IL-12 and IL-18 induced a much stronger antiviral effect (nearly 10 times higher) than what would be expected from the additive action of both cytokines.

Data shown in Fig. 3 indicate that IL-12 is more effective than IL-18 for the control of VV infection and when both cytokines are expressed from rVVs they induced a synergistic antiviral effect.

Pattern of cytokines induced after expression of IL-18 and IL-12 from rVVs

Since the delivery of a cytokine from rVV can affect the expression of other cytokines, we next analysed the levels of Th1 cytokines (IL-12, IL-18 and IFN-γ) induced in serum and spleen. Mice were inoculated with rVVs expressing IL-12 and IL-18, as described in Fig. 3, and the cytokine levels in serum and spleen were measured at various times p.i. As observed in Fig. 4, at shorter times p.i. (6 h), IFN-γ levels in the serum were elevated but were similar between groups III and IV. At later times p.i. (16 and 24 h), mice receiving both viruses showed the highest difference in levels of IFN-γ (nearly a 2-fold increase) with respect to the group inoculated with rVVIL-12. Thereafter, no appreciable differences were observed between the groups. The production of high levels of IFN-γ by co-expression of IL-12 and IL-18 was also supported by the findings obtained in spleen homogenates at 1 day p.i.; however, at 2 and 3 days p.i., the differences between the groups were less evident (Fig. 4).

Surprisingly, when the levels of IL-12 and IL-18 were measured at early times p.i. (up to 1 day), the levels of both cytokines in group IV (rVVIL-12 + rVVIL-18) were significantly lower compared to the levels detected in group III (rVVIL-12) or group II (rVVIL-18) (Fig. 4). Mice of groups II and III had the highest levels of IL-18 and IL-12, respectively (in both serum and spleen), but in group IV those levels were greatly diminished compared to group II or III, respectively ($P<0{\cdot}01$ performed in individual spleen samples). This effect was detected at short times p.i. (6–24 h p.i.), when differences in levels of virus replication (as index of luciferase activity) between the different groups were not significant. Results from Fig. 4 indicated that expression of IL-12 and IL-18 from rVV induced a relatively increased level of IFN-γ and suggested that expression of IL-18 and IL-12 may be negatively regulated when both cytokines are delivered from rVV. This might be a protective mechanism

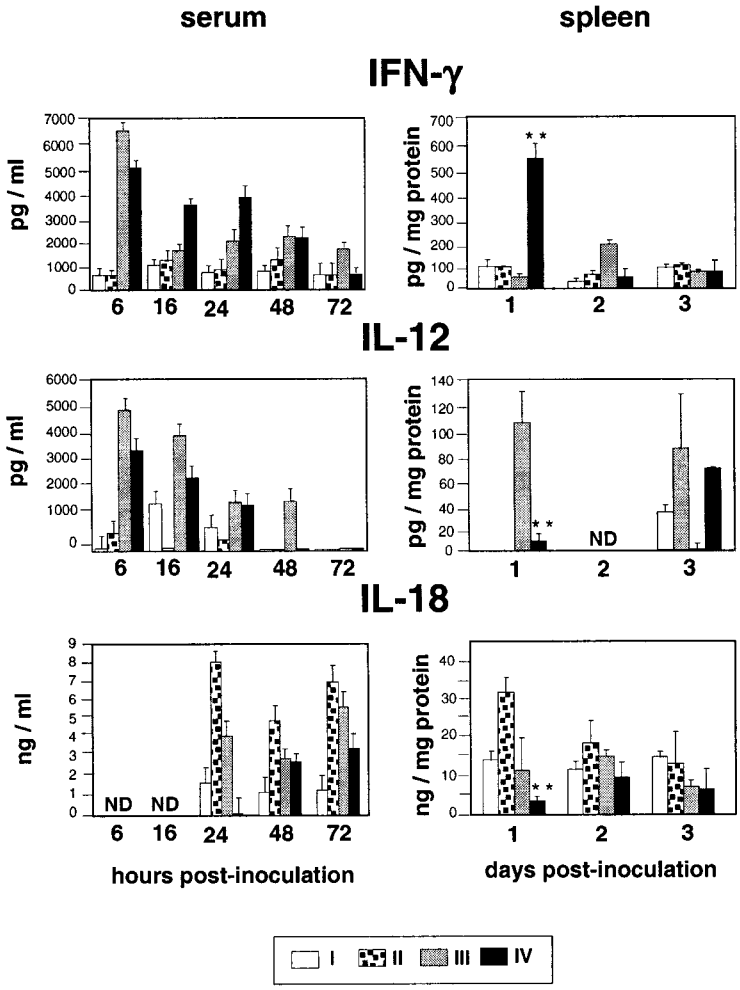

Fig. 4. Kinetics of cytokine responses after expression of IL-18 and IL-12 from rVVs. Samples of serum and spleen from the groups of mice of the experiment depicted in Fig. 3 were obtained at the times indicated. Serum determinations of IFN-γ, IL-12 and IL-18 were performed in pooled samples from three to four mice per group by sandwich ELISA. Data represented are mean values $\pm$SD of two ELISA determinations. Levels of the cytokines in cleared spleen homogenates were performed in individual samples and related to the total protein in the spleen homogenates. Data represented are mean values $\pm$SD for three to four mice per group. Basal levels for each cytokine detected in serum and spleen homogenate samples from naive mice have been subtracted from the values found in the experimental groups; the values were <25 μg protein mg^{-1} for IL-12 and IFN-γ and 6 ng protein mg^{-1} for IL-18. In serum, values <100 pg ml^{-1} were found for the three cytokines tested. Cytokine levels significantly different with respect to those found in the other groups are denoted as ** ($P<0{\cdot}01$).

directed to regulate the production of undesirable quantities of IFN-γ that may otherwise cause toxic effects.

The combined action of IL-12 and IL-18 delivered from rVVs results in enhanced specific humoral and cellular immune responses to VV

Our next aim was to study the consequences that co-expression of IL-12 and IL-18 from rVVs might have on induction of specific anti-VV humoral and cellular immune responses. Mice were inoculated intraperitoneally with rVVs expressing IL-12 or IL-18, following the scheme depicted in Fig. 5. At 10 days p.i., the $CD8^+$ T cell immune response against VV was evaluated by a modified ELISPOT assay that quantifies IFN-γ-secreting $CD8^+$ T cells (Fig. 5A) (Gherardi *et al.*, 1999) and, at 14 days p.i., the specific anti-VV IgGs in the sera were determined by ELISA (Fig. 5B). As shown in Fig. 5(A), mice inoculated with both rVVIL-12 and rVVIL-18 (group IV) triggered an enhanced specific $CD8^+$ T cell response against VV when compared to mice inoculated with rVVIL-18 (group II) ($P<0{\cdot}01$) or rVVIL-12 (group III) ($P<0{\cdot}01$) alone. The increment in IFN-γ-secreting cells induced independently by each cytokine was 1·6 (rVVIL-18) or 2·7 (rVVIL-12) times higher than in the control group. Thus, the 4-fold increase observed in the specific $CD8^+$ T cell response of group IV is in line with an additive effect of both cytokines. When the levels of specific IgG subclasses induced in the different groups were evaluated 14 days after rVV inoculation, group IV gave the highest ratio (IgG2a : IgG1) of specific IgGs (Fig. 5B). The increment observed in this group was significantly different ($P<0{\cdot}05$) with respect to the response observed in the other three groups. Based on the findings of Fig. 5, we conclude that the concerted action of both IL-12 and IL-18 delivery in combination by rVVs is more effective in promoting a specific Th1 type of immune response to VV than when each cytokine was delivered individually by the recombinant virus.

T cells are involved in the IL-12 + IL-18 synergism to reduce VV infection

To evaluate the involvement of T cells in the resolution of VV infection, we inoculated SCID BALB/c and normal BALB/c mice with $rVVHA^-$ or rVVIL-12 + rVVIL-18 (groups I and IV, Fig. 3). At 3 days p.i., animals were sacrificed and luciferase activity was measured in the ovaries. In both types of mice, the differences between $rVVHA^-$- and rVVIL12 + rVVIL-18-inoculated animals were significantly different ($P<0{\cdot}05$). However, the levels of luciferase in

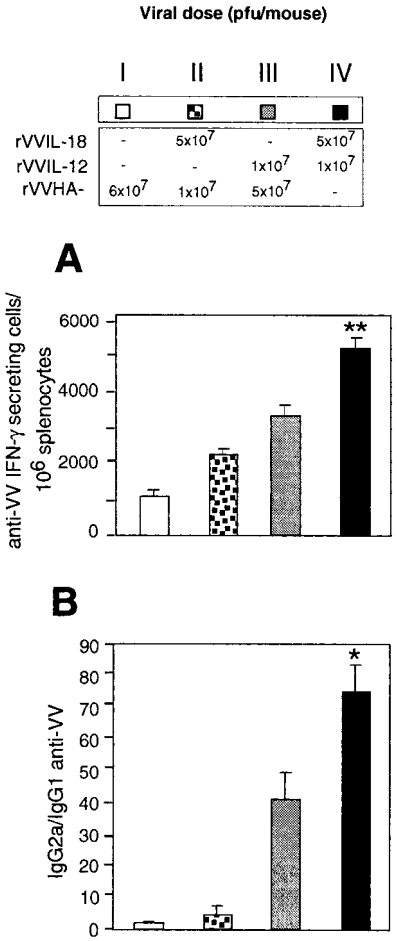

Fig. 5. Specific cellular and humoral anti-VV immune responses following expression of IL-12 and IL-18 from rVVs. BALB/c mice were inoculated following the scheme depicted in the top of the figure. (A) At 10 days after the immunization, the specific cellular immune response against VV was evaluated by counting the number of splenic-specific IFN-γ-secreting $CD8^+$ T cells by an ELISPOT assay. Bars represent mean values $\pm$SD of triplicate cell cultures from four mice per group. Similar results were obtained in three independent experiments. Significant differences with respect to values found in the other groups are denoted as ** ($P<0{\cdot}01$). (B) Mice were inoculated as in (A) and at 14 days p.i. individual serum samples were obtained. Anti-VV IgG1 and IgG2a antibody titres were calculated in individual samples as the inverse $\log_2$ dilution of sera that gave $A_{492}>0{\cdot}1$. Bars represented the mean IgG2a : IgG1 ratio $\pm$SD of four mice per group. Significant differences with respect to values found in the other groups are denoted as * ($P<0{\cdot}05$).

immune-competent BALB/c mice were 100-fold lower in mice given rVVIL-12 + rVVIL-18 than in mice inoculated with the control virus (Fig. 6A, right) and, in SCID mice, this difference was reduced greatly (by 7-fold) (Fig. 6A, left). To examine if, under these conditions, the levels of induced IFN-γ are related to differences in VV clearance, we measured IFN-γ levels in serum at 1 day p.i. and in supernatants of cell cultures from splenocytes obtained 3 days post-rVV infection from the two groups of infected mice. Differences in the levels of IFN-γ (Fig. 6B) between the two groups were of the same magnitude, regardless of the type of mouse strain (normal BALB/c or SCID BALB/c) used. The experiments of Fig. 6 revealed that in the absence of T cells it is possible to establish an anti-VV protective response by the combined action of IL-12 and IL-18, but the antiviral action displayed by IL-12 + IL-18 was minor with respect to that developed in immune-competent mice.

Participation of NK cells in the combined action of IL-12 + IL-18 against VV infection

Next, we analysed the role of NK cells in the synergistic antiviral effect of IL-12 + IL-18. To this aim, NK cells were depleted with the antibody PK136. This antibody is specific for the NK-1.1 surface antigen expressed on NK cells of C57BL/6 mice but not of BALB/c mice. Thus, we performed a preliminary experiment similar to that done with the BALB/c mice, as described in Fig. 3. We found that VV infection in C57BL/6 mice (Fig. 7) given rVVIL-12 + rVVIL-18 was similar to that observed previously in BALB/c mice. In C57BL/6 mice inoculated with rVVIL-12 + rVVIL-18 (group IV), levels of luciferase were nearly 50 (2 days p.i.) and 100 (3 days p.i.) times lower than that in control mice. In rVVIL-12-inoculated mice (group III), however, luciferase activity at 2 days p.i. and 3 days p.i. was 25 and 10 times lower than that in control mice. In rVVIL-18-inoculated mice (group II), there is a minor antiviral effect, nearly a 2·5- and 1·5-fold drop in luciferase activity at 2 and 3 days p.i., respectively. We also found that 3 days p.i., differences between groups III (rVVIL-12) and IV (rVVIL-12 + rVVIL-18) were significantly different ($P<0{\cdot}05$). Thus, in C57BL/6 mice, we found that co-delivery of IL-12 and IL-18 induced a profound reduction in VV replication, indicating an antiviral synergism between both cytokines.

To perform NK depletion experiments, 1 day before virus inoculation (day -1), C57BL/6 mice were inoculated intraperitoneally with PK136 antibody or control sera. Thereafter (day 0), the different rVVs were inoculated following the scheme described in Fig. 8. At 3 days p.i., ovaries were obtained for luciferase determination. Fig. 8 shows that in groups I, II and III, levels of luciferase are equal, regardless of the depletion of NK cells, whereas in mice inoculated with rVVIL-12 + rVVIL-18 (group IV), the elimination of NK cells abrogates the synergism between both cytokines. Indeed, in NK-depleted animals (group IV), the levels of luciferase were similar to the ones found in group III. Thus, at 3 days p.i., significant differences ($P<0{\cdot}05$) were observed in group IV between antibody- and mock-treated mice and the differences between groups III and IV ($P<0{\cdot}01$) in mock-treated mice disappeared in the presence of antibody. These data suggest that NK cells are mediators, at least in part, of the required synergism between IL-12 and IL-18 for VV clearance in ovaries.

After IFN-γ determinations in serum (6 h) and spleens (24 h) of C57BL/6 mice from groups I and IV, we found that

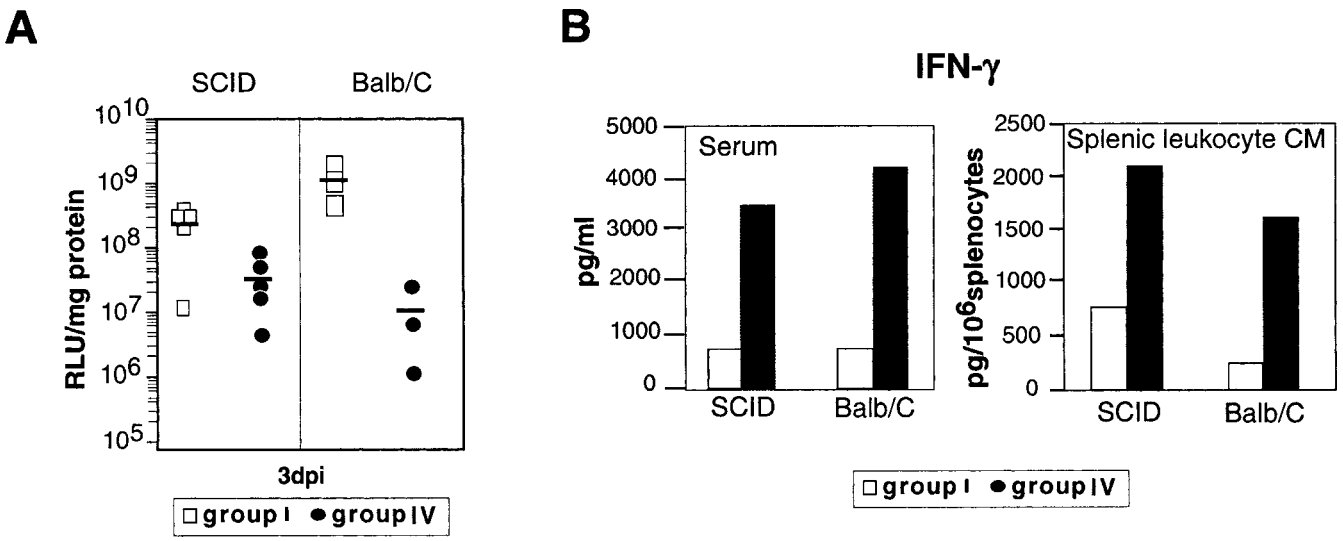

Fig. 6. Relevance of the specific anti-VV T cell immune responses in the synergism of IL-12 and IL-18 against virus infection. BALB/c and SCID mice were inoculated intraperitoneally with control rVVHA$^-$ or rVVIL-12+rVVIL-18, as in groups I and IV (Figs 2 and 4). (A) At 3 days p.i., virus replication was evaluated by measuring luciferase activity in ovary tissue from three to five mice per group (left panel). Each symbol corresponds to an individual mouse and the thick bars indicate the average value. (B) At 1 day p.i., serum samples of the different groups were obtained and IFN-γ levels were calculated by a sandwich ELISA (left panel). Bars represent values obtained in pooled serum samples from four mice. At 3 days p.i., splenocytes from the different groups were obtained and cultured, as described in Methods, and after 24 h IFN-γ levels were evaluated in cell culture supernatants (right panel).

treatment with the anti-NK antibody did not significantly reduce the levels of IFN-γ (data not shown).

DISCUSSION

Expression of cytokines by rVVs has been proven as an effective research approach to evaluate the role of several immune modulators in host responses against virus infection. Using rVVs expressing IL-18 or IL-12, we have addressed the involvement and interaction of both cytokines in the modulation of the antiviral immune response and virus resolution during VV infection in the mouse. Previous reports have documented that IL-18 acts in functional synergy with IL-12. Thus, combinations of both cytokines *in vitro* induce a synergistic production of IFN-γ in T, B and NK cells (Okamura *et al.*, 1995; Kohno *et al.*, 1997; Tomura *et al.*, 1998; Micallef *et al.*, 1996) as well as in APCs (Fukao *et al.*, 2000; Munder *et al.*, 1998). *In vivo*, synergistic effects in tumour models (Osaki *et al.*, 1998; Coughlin *et al.*, 1998) and preventive functions in an allergic asthma mouse model (Hofstra *et al.*, 1998) have also been described. Despite the protective roles of IL-12 and IL-18 in host defence mechanisms against pathogens (Nakanishi *et al.*, 2001), there are few reports addressing the interaction *in vivo* between IL-12 and IL-18 against pathogens (Qureshi *et al.*, 1999; Cai *et al.*, 2000; Harandi *et al.*, 2001). Here, we have shown that enhanced VV clearance in mice co-inoculated with rVVIL-12 and rVVIL-18 implies that both cytokines act synergistically in the clearance of the virus of the mouse involving NK cells and enhancing the specific CD8$^+$ T cell and Th1 antiviral immune responses. Levels of virus expression in target tissues (ovaries and spleen) at the peak of virus replication when both cytokines were co-delivered were close to 300-fold lower than that of the control and should not merely result from the additive inhibition observed when delivered alone (see Fig. 2). Hence, our data support the idea that a synergism in the interaction of both cytokines is occurring. We observed significantly lower antiviral effects of IL-18 relative to IL-12 when delivered from rVV. Several activities encoded by VV genes could contribute to evade IL-18 function, like VV ORF B13R acting on caspase-1 (Kettle *et al.*, 1997), expression of IL-18-binding protein (IL-18 BP) (Smith *et al.*, 2000), VV ORF A52R (Bowie *et al.*, 2000), which blocks the IL-18-dependent NF-κB activation, and C12L expressed by the WR strain with amino acid similarities to IL-18 BP (Symons *et al.*, 2002). Expression of IL-12 or IL-18 from rVVs does not affect virus replication *in vitro*. Thus, the attenuation observed *in vivo* of VV associated with the expression of IL-12 and IL-18 is probably an indirect effect due to secondary induction of immune components that eliminate VV-infected cells. IFN-γ is well established as a key mediator in VV clearance (Ramshaw *et al.*, 1997) and is involved directly in some of the biological actions of IL-12 and IL-18. Our findings, however, suggest that the synergism in the action of both cytokines in VV clearance is not related to overproduction of IFN-γ. In both serum and spleen, the

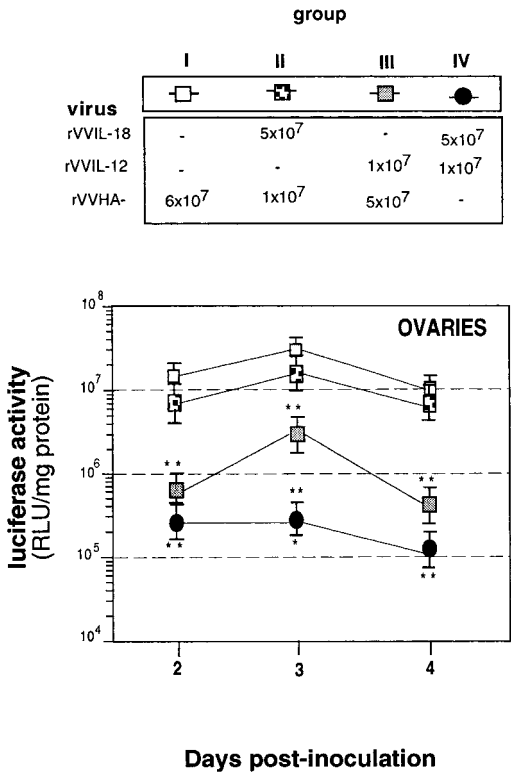

Fig. 7. Expression of IL-12 and IL-18 from rVVs in C57BL/6 mice. Mice were inoculated intraperitoneally with the doses and combination of viruses depicted in the upper part of the figure to a final dose of rVV expressing luciferase of 6×10^7 p.f.u. At the times indicated, ovaries from four mice were processed and luciferase activity measured, as described in Methods, and related to protein content. Each point represents mean values from four mice $\pm$SD. Significant differences relative to control group (group I) are denoted as ** ($P<0{\cdot}01$); significant differences between groups III and IV are denoted as * ($P<0{\cdot}05$).

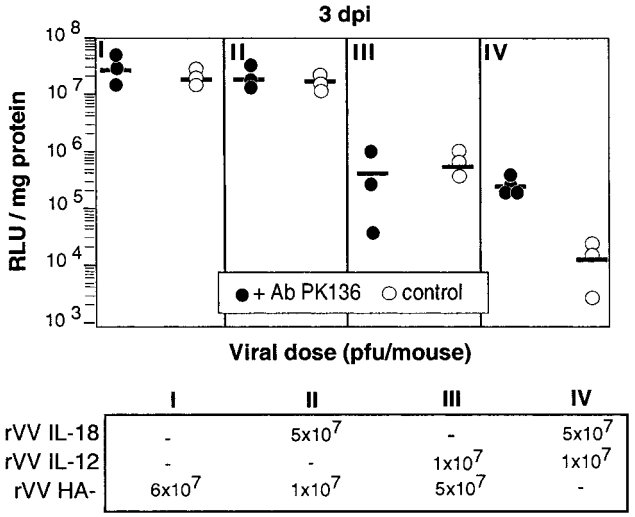

Fig. 8. Role of NK cells in the IL-12 and IL-18 action against VV infection. C57BL/6 mice were depleted of NK cells by intraperitoneal inoculation with the PK136 antibody or treated with normal control serum and 24 h after the different combinations of rVVs indicated were inoculated intraperitoneally. At 3 days p.i., luciferase activity was measured in ovaries from the different groups; the individual values of each group and its corresponding mean are shown by a thick bar.

highest levels of IFN-γ were produced in mice infected with rVVIL-12 and rVVIL-18 in combination, but the observed increase in IFN-γ appears to be an additive rather than a synergistic effect. However, in the spleen at 1 day p.i., an apparent synergism was observed but it did not last through the following days. Indeed, IFN-γ seems not to be the main mediator in the antiviral effect of co-delivery of IL-18 and IL-12 in both immunosuppressed mice and mice devoid of NK cells. Thus, levels of IFN-γ in serum and spleen supernatants were comparable to those in control mice (BALB/c mice or mice given control antibody, respectively). However, the protective effect against VV infection by the co-delivery of IL-12 and IL-18 was very diminished in SCID or NK-depleted mice. These results concur with a recent report showing that endogenous IL-12 is more important than IFN-γ for VV clearance (van den Broek *et al.*, 2000). Since the combination of IL-12 and IL-18 may cause severe and synergistic toxic effects involving IFN-γ dependent and independent mechanisms (Nakamura *et al.*, 2000), it is suggested that when IL-12 and IL-18 are co-delivered, protective mechanisms prevent the production of excessive quantities of IFN-γ that may otherwise cause toxic effects. Nevertheless, there are reports suggesting a direct correlation between the synergistic action of IL-12 and IL-18 and increased levels of IFN-γ in a tumour mouse model (Osaki *et al.*, 1998) and in a cryptococcal infection model (Qureshi *et al.*, 1999). Although it is known that both cytokines enhance receptor expression reciprocally, we have observed in our system a cross-talk between IL-12 and IL-18 that negatively regulates the induction of each other when co-delivered from rVVs. In mice inoculated with rVVIL-12 and rVVIL-18, levels of both cytokines in serum and spleen were greatly reduced compared to mice given rVVIL-18 or rVVIL-12 alone, in line with IL-12 properties (Gherardi *et al.*, 2001; Yoshimoto *et al.*, 1998; Kim *et al.*, 2001). Moreover, we have found that in mice inoculated with a dose of 10^8 p.f.u. per mouse of rVVIL-18, serum and spleen contents of IL-12 were lower compared to control mice (data not shown). These data agree with other studies in bacterial infections, which suggest a regulatory mechanism to balance uncontrolled IL-12 production (Dybing *et al.*, 1999; Bohn *et al.*, 1998).

Interestingly, some authors (van den Broek *et al.*, 2000) have found that high and low susceptibility to VV infection correlate with the absence and presence of CTL responses in IL-$12^{-/-}$ and IFN-$\gamma^{-/-}$ mice, respectively. We have found that co-delivery of both cytokines by rVVs induces an enhanced specific antiviral $CD8^+$ T cell response when compared to mice given rVVIL-12 or rVVIL-18 alone. We

also found that rVVIL-12 + rVVIL-18 treatment favours the increment of the specific Th1 : Th2 (IgG2a : IgG1) ratio. The importance of T cells on IL-12 + IL-18-mediated VV clearance was demonstrated further by experiments with SCID mice. Previous studies have demonstrated that IL-18 plays an important role in the generation of type I effector $CD8^+$ T cells in a $CD4^+$ T cell-dependent manner (Okamoto *et al.*, 1999) and suggested that functional maturation of $CD8^+$ T cells is differentially regulated by IL-18 and IL-12. Both regulatory mechanisms might be operating in mice inoculated with rVVs co-expressing both cytokines. Moreover, independent and synergistic effects of IL-18 and IL-12 in augmenting cytotoxic T lymphocyte responses and IFN-γ production in ageing have also been demonstrated (Zhang *et al.*, 2001).

NK cells play a critical role in innate immunity against pathogens, virus-infected cells and tumours through MHC-unrestricted cytotoxicity and production of cytokines. Positive synergy has been demonstrated between IL-12 and IL-18 in NK proliferation, cytotoxicity and IFN-γ production (Tomura *et al.*, 1998; Lauwerys *et al.*, 1999) and in mice lacking both IL-12 and IL-18, NK activity and Th1 responses were impaired further (Takeda *et al.*, 1998). Our findings indicate that NK cell depletion affected the IL-12/IL-18 synergism in the anti-VV protective effect in ovaries, suggesting an important role for NK cells in such synergism. Indeed, this unique effect on NK cells relies on the combined action of both cytokines, as rVV replication is not affected after NK depletion in mice given control rVV, rVVIL-12 or rVVIL-18 alone. To this end, it has been described recently that NK cells derived in the presence of IL-12 and IL-18 displayed strong and unique cytotoxicity, involving the induction of apoptosis and higher expression of perforin compared to NK cells derived in the presence of IL-12 or IL-15 (Lauwerys *et al.*, 2000). Our data, and those reported by others (Mahalingam *et al.*, 1999), show that elimination of NK cells had a negligible effect on the control of VV. However, the elimination of NK cells exacerbates the infection with chemokine-encoding rVV, suggesting that NK depletion only affects rVV replication when cytokines or chemokines influencing the antiviral effect of NK functions are expressed from rVVs.

In conclusion, we have described in the mouse model that when IL-12 and IL-18 are co-delivered from rVVs, a synergism in the protective antiviral effect occurs. T and NK cells are involved in such an effect: SCID mice showed a less pronounced clearance of the recombinant virus compared to normal BALB/c mice and NK depletion abrogated the synergism in clearance of the virus. Importantly, besides virus clearance, a bias of the specific anti-VV immune response towards a Th1 type was obtained, as mice inoculated with rVVIL-12 + rVVIL-18 showed an enhanced specific $CD8^+$ T cell response as well as an incremented IgG2a : IgG1 ratio. These findings are of interest for the design of both therapeutic and prophylactic vaccines based on poxvirus vectors.

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